U. S. NAVAL MEDICAL SCHOOL
Serology.

SEROLOGY MANUAL

U.S. NAVAL MEDICAL SCHOOL

ERRATA SHEET

PAGE 3: Paragraph 2, line 7. Insert In before "new cardiolipin antigens --". Delete the comma after "lecithin."

PAGE 4: Delete the paragraph on the T.P.I. test and insert the following:

The TPI (Treponema pallidum immobilization) test is performed by placing the patient's serum in a test tube with living spirochetes and complement. After 18 hours incubation at 34°C. in an atmosphere free of oxygen, the spirochetes will be immobilized by syphilitic serum but will be actively motile in normal serum. The spirochetes from each tube are examined by darkfield microscopy to determine the percentage of motile organisms.

The test is a complicated procedure with numerous problems, one of which is the procurement of satisfactory rabbits for the maintenance of the T. pallidum (Nichol's strain) through successive animal passage. Because of the problems and the expense of the TPI procedure and the limited number of trained personnel, it probably will never become a routine laboratory test. It is considered of value along with the S.T.S. (Standard Tests for Syphilis) and clinical findings in the resolution of diagnostic problems.

The immobilization antibody is believed to be different than reagin. It arises later in the course of the disease. In early syphilis the S.T.S. may be positive and the TPI negative. If treatment is instituted after the S.T.S. and the TPI are both positive, the S.T.S. will revert to negative first. The TPI may never become negative, or if treatment was instituted early enough, there will be a lag of several months after the S.T.S. are negative before the TPI also becomes negative. Therefore, it is possible for the TPI to be positive when the S.T.S. are negative.

The diagnosis should not be based on the results of any one test and should be considered along with the clinical findings.

PAGE 4: Delete the paragraph on TPA and insert the following:

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is examined under the microscope. The production of clusters of treponemes is observed in the presence of positive serum. The size of the aggregates varies with the amount of antibody present so some zonal reactions may occur. Research is continuing to determine the best preservative for treponemal antigen for this test. Some solutions permit spontaneous clumping of the treponemes on storage.

PAGE 25: In Supplementary Test #1. Under 2, insert 0.05 instead of "0.5" ml.

PAGE 28: Last line under paragraph "Determination of Kahn Units", should be (1:64 dilution).

PAGE 44: Under paragraph numbered 5, delete sentences in parentheses following Table 2 and substitute as follows:

(For speed and accuracy in the addition of saline a 5.0 ml. (graduated serological) pipette should be used. The readings of the pipette upon successive deliveries of saline starting with tube 2 through 4 are: 0.5, 1.5, 3.0. Refill the pipette, add 2.0 ml. to tube 5 and 0.5 ml. to the rest of the tubes. In serological technic, it is not good practice to use the last 0.5 ml. of fluid at the tip of the pipette.)

PAGE 46: Paragraph 9, add the following after Table 4:

Successive readings of the 1.0 ml. (graduated serological) delivery pipette will be 0.2, 0.45, 0.75. Refill the pipette for delivery readings of 0.35, 0.75. Refill the pipette for delivery readings of 0.45 and 0.95.

PAGE 46: Paragraph 11, add: For speed in the addition of 1.7 ml. of saline to each tube, a 10 ml. (graduated serological) pipette is used. This is the only time in the Kolmer test where the use of a 10 ml. pipette is advised. Readings upon delivery of 1.7 ml. of saline to successive tubes will be 1.7, 3.4, 5.1, 6.8, 8.5. Refill the pipette and repeat.

PAGE 46: Paragraph 12, add the following after Table 5:

For addition of saline a 5.0 ml. (graduated serological) pipette is employed. The readings of the pipette on progressive delivery of saline to the respective tubes will be 1.3, 2.6, 3.8. Refill the pipette and deliver 1.2 to tube 4, next reading for tube 5 and successive deliveries will be 2.3, 3.4, 4.4. Refill the pipette to deliver 2.5 ml. to tube 8.

# SEROLOGY

1959


NAVAL MEDICAL SCHOOL
NATIONAL NAVAL MEDICAL CENTER
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# PREFACE

This manual was prepared and edited by the staff of the United States Naval Medical School. It is intended primarily as a textbook for laboratory students and as a reference guide for clinical laboratory technicians of the Medical Department, United States Navy.

Proven methods, currently in use at the Naval Medical School, are herein presented in easily available form. Accuracy and simplicity are the primary objectives. The procedures outlined are for use in the field as well as naval hospital laboratories.

The technician's knowledge and skill in Serology, accurately applied, is of inestimable value to the clinician in charge of patient care.

L. J. POPE
CAPT MC USN
Commanding Officer
U.S. Naval Medical School
National Naval Medical Center
Bethesda, Maryland

# SEROLOGY

CURRENT REVISION BY

FRANCES SPEAR, A.B., M.S., LCDR, MSC, USN

Officer in Charge, Serology Laboratory

## REFERENCES

Reference may be made to the following for additional information:

Davidsohn, I; Stern, K; and Kashiwagi, C.: "The Differential Test for Infectious Mononucleosis," reprinted from "Abraham Levinson Anniversary Volume Studies in Pediatrics and Medical History," Froben Press, Inc., New York, 1949.

Kahn, R. L.: "Serology with Lipid Antigen," Williams & Wilkins Co., Baltimore, 1950.

Manual, "Serologic Tests for Syphilis," U. S. Department of Health, Education, and Welfare; Bureau of State Services; Division of Special Health Services; Venereal Disease Program; United States Government Printing Office, 1955.

Kolmer, J. A.; Spaulding, E. H.; and Robinson, H. W. "Approved Laboratory Technic, "Appleton Century Crofts, New York City, 1951.

## CONTENTS

## SECTION I

## SEROLOGY

Serology is a laboratory means of measuring the potential protective antibodies in the individual or patient. Serological technics also provide diagnostic methods for the identification of infectious agents as well as identifying antigens or antibodies not related to disease.

Antibodies produced in response to a simple antigen may bring about a variety of observable results depending on technic and the antigen employed.

PRECIPITATION: Soluble antigens may be precipitated through the action of antibody. The greatest amount of precipitation will be noted in the region where antigen and antibody are present in optimal or immunological equivalent proportions. In regions of antibody excess with reference to the amount of antigen present, or conversely where antigen is present in excess with reference to antibody there will be no visible precipitate. The table below shows optimal proportions when various dilutions of antigen are tested against various dilutions of serum containing antibody.

Table 1. Optimum Antigen-Antibody Ratio

| Serum Antibody Dilution | Antigen Dilution | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Undiluted | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 | 1:1024 |
| Undiluted | 4+ | 4+ | 4+ | 3+ | 3+ | 2+ | + | + | + | | |
| 1:2 | 2+ | 4+ | 4+ | 3+ | 3+ | 2+ | + | + | + | | |
| 1:4 | | + | + | 3+ | 3+ | 2+ | + | + | + | | |
| 1:8 | | | | + | 3+ | 3+ | 2+ | + | + | | |
| 1:16 | | | | | + | 2+ | 3+ | + | + | | |
| 1:32 | | | | | | + | 2+ | 2+ | + | | |
| 1:64 | | | | | | | | + | 2+ | + | |
| 1:128 | | | | | | | | | + | + | + |
| 1:256 | | | | | | | | | - | - | - |

AGGLUTINATION: Agglutination is similar to precipitation in that aggregates are formed but in this instance the antigen is particulate, such as bacteria or blood cells.

LYSIS: Antigens, such as bacteria or blood cells may be lysed if antibody plus complement is present. In this process a cell is caused to lose its contents or is broken.

SENSITIZATION: Antibody may coat bacteria or blood cells. By this means the

process of phagocytosis is hastened, sometimes called opsonization. Sensitized (antibody coated) red cells are hemolyzed more easily, as in cases of erythroblastotic babies.

COMPLEMENT FIXATION: Antigen and antibody may combine and by so doing absorb complement. This phenomenon is called complement fixation.

nization. Sensitized
1 cases of erythroblas-

bine and by so doing ab-
fixation.

## SEROLOGIC TESTS FOR SYPHILIS

Serodiagnostic tests for syphilis are of two types, the complement fixation and the precipitation tests. The various precipitation tests are also spoken of as flocculation tests. Both are based upon the ability of the antigens used to detect the presence of an antibody-like substance, known as reagin. The term, syphilitic reagin, is used to designate the antibodies that result from an infection by Treponema pallidum. These antibodies are specific and are detectable by the various serodiagnostic precipitation tests because the antibody or reagin produces changes in the dispersion of the antigen lipids. These changes are manifested by the formation of visible aggregates, the sizes of which are roughly proportional to the amount of reagin present.

There are two types of antigens used in the various serodiagnostic tests. The "older antigens" frequently referred to as the "extract antigens" are made from the alcoholic extract of beef heart. The ether soluble lipids have been previously removed from the beef heart by repeated extractions with ether. Cholesterol is added to the alcoholic extract. The "extract antigens" contain in addition to cholesterol, the alcohol soluble, ether insoluble lipids of beef heart, as well as traces of various other substances that are insoluble in ether and soluble in alcohol. In the new or cardiolipin antigens, the cardiolipids and lecithin are extracted from the beef heart and purified. Cholesterol is added to the alcoholic mixture of the cardiolipids and lecithin. The authors of many of the various precipitation and complement fixation tests have modified their procedures so that the test is now performed with cardiolipin antigen.

The Kahn precipitation test has shown a consistent record of extraodinarily high specificity (freedom from false positive reactions) in serologic evaluation studies. It has been the official serologic test for syphilis in the Navy since 1925 and has been found to be satisfactory ashore and afloat in all climates. The antigen used by all naval medical activities is made at the United States Naval Medical School where it is carefully standardized and finally approved by Kahn's laboratory before it is released for use. This policy has produced a high degree of standardization of serodiagnostic results throughout the naval service.

The technics of various tests are given in the following pages. The directions must be followed rigidly if standard results are to be obtained.

## TESTS EMPLOYING TREPONEMAL ANTIGENS

The tests for syphilis employing antigens of Treponema pallidum are not included in this manual. However, since it is important that the technician be aware of their existence, the following descriptions are given.

The TPI (Treponema pallidum immobilization) test is performed by placing the patient's serum in a test tube with living spirochetes and complement. After 18 hours incubation at 34°C. in an atmosphere free of oxygen, the spirochetes will be immobilized by syphilitic serum but will be actively motile in normal serum. The spirochetes from each tube are examined by darkfield microscopy to determine the percentage of motile organisms.

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The T.P.I.A. (Treponema pallidum Immune Adherence) test utilizes the patient's serum, Treponema pallidum, complement, and the patient's own or group "O" red blood cells incubated together at 37°C. With syphilitic serum, the treponemes adhere

to the red blood cells, but with normal serum, do not. This phenomenon is observed when the tubes are spun sufficiently to sediment the red blood cells. If the supernate contains a number of treponemes on darkfield examination, the test is negative.

The T.P.C.F. (Treponema pallidum Complement Fixation) test represents a modification of the one-fifth volume Kolmer complement fixation test for syphilis. Earlier findings suggest that the T.P.C.F. test identifies an antibody other than reagin or immobilizing antibody and may be of value in the detection of syphilis.

The T.P.M.B. (Treponema pallidum Methylene Blue) test utilizes the phenomenom, by which virulent T. pallida (normally stained by methylene blue) becomes resistant to the dye following incubation with positive serum and complement. The differentiation is easily detected by darkfield microscopy. This reaction provides the basis for a specific test for syphilis.

The unique advantage of these tests is that they employ the specific antigens of Treponema pallidum in an in vitro reaction.

These tests are under study and evaluation in research laboratories and may one day become a part of the battery of tests in clinical laboratories.

When a culture media is perfected on which the organism can be grown, rather than in the rabbit, a great impetus in this direction will be assured.

## SECTION II

## TECHNIQUE OF VENIPUCTURE

The technique of venipuncture can be readily demonstrated by a series of drawings as shown in the illustrations on the opposite page. The following outline of this technique may be of additional value to the student:

1. Assemble the equipment. Label the tubes.

2. Place a tourniquet around the patient's arm above the elbow tightly enough to check venous circulation, but not so tightly as to stop arterial flow. Request the patient to open and close the fist.

3. By inspection and palpation, locate the desired vein, determine the direction of its course, and estimate its size and depth.

4. Cleanse the skin over the selected vein. Place a sponge on the arm above the site of puncture. Allow the alcohol to dry before making the puncture to prevent tattooing the skin with alcohol. Do not contaminate the area after cleaning. (If blood is drawn for culture, use mild tincture of iodine or merthiolate. If blood is for a Bogen's test, use a nonalcoholic antiseptic, such as aqueous solution of merthiolate.)

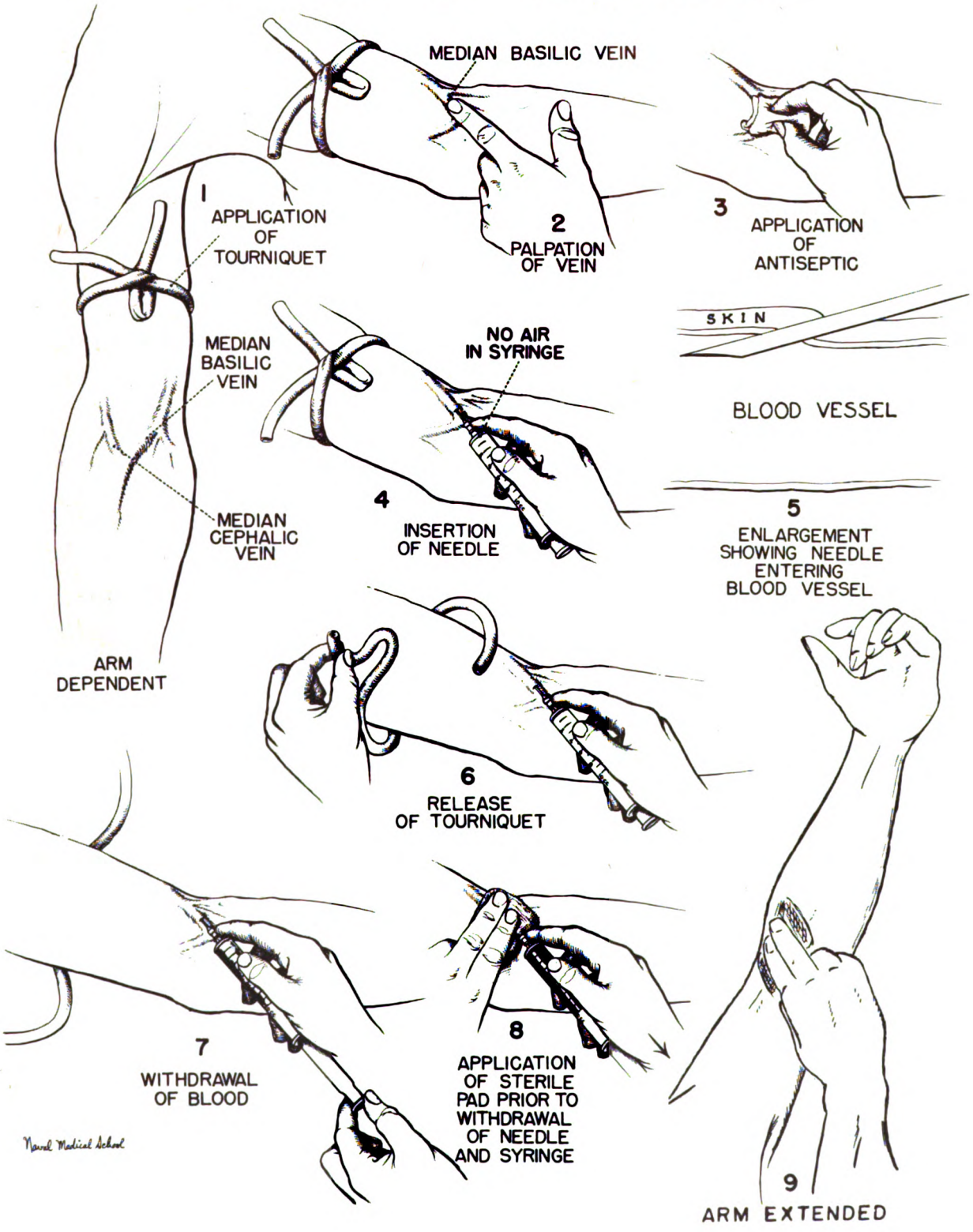
5. Fit the needle to the syringe while the alcohol is drying. The plunger must match the syringe and must be held tightly in the cylinder at all times in order to prevent injection of air into the vein.

6. Have the patient "make a fist" and straighten the arm. Frequently it is helpful to have the patient place the clenched fist of the other hand under the elbow to straighten the arm.

7. Grasp the syringe in the right hand (left for left-handed person) and place forefinger on the needle to guide it. With the needle pointing in the direction of the course of the vein, make the venipuncture. This operation may be considered in two steps: First the skin is broken, secondly the vein is entered. A slight "give" indicates the vein is punctured. In vigorous individuals, blood will appear spontaneously. If pressure in the vein is low, the plunger may have to be withdrawn slightly to draw out the blood. Slide the needle a short distance (one-fourth to one-half inch) farther into the vein to prevent the needle from coming out while the blood is being withdrawn. Take care not to go through the other side of the vein. Use the left hand to pull the plunger while steadying the syringe with the right hand.

8. Release the tourniquet. Place a sponge over the site of puncture and withdraw

# TECHNIQUE OF VENIPUNCTURE



the needle. Instruct the patient to open the hand and press the sponge over the wound for 2 to 3 minutes. (For hematocrits and fragilities the tourniquet is removed as soon as blood appears in the syringe.)

9. Remove the needle from the syringe and run blood down the side of the tube, using gentle pressure on the plunger to avoid foam and rupture of the cells. In filling the tubes with marked measurements, the needle is preferably left on and the plunger pulled back slightly just as the mark is reached to prevent more blood from entering the tube.

10. The oxalate and citrate containers are filled before the tubes for clotted blood specimens.

11. All syringes, needles, lancets, or other instruments used for the collection of blood specimens must be sterile. Sterilization by dry heat or autoclaving is preferable and recommended, but boiling for a minimum of 5 minutes is satisfactory. This is necessary to prevent the possible transmission of homologous serum jaundice.

12. The following points may be of help in difficult venipunctures:

a. Veins may be made more prominent and easier to enter by allowing the arm to hang down for 2 to 3 minutes, by massaging toward the body, and by smartly but lightly slapping the site of puncture.

b. In some persons veins become indistinct after the tourniquet has been on 3 or 4 minutes.

c. Young and vigorous persons usually have elastic veins well filled with blood so that entrance to the vein is easy and the blood flow is good. Elderly or weak persons may have calcified veins which are hard to enter or of such low pressure that veins collapse under pressure of the needle.

d. Occasionally the best vein is located in the hand, leg, or foot. These areas are more sensitive, however, and the veins usually less firmly anchored than those of the arm.

e. Rolling veins may be held by placing the thumb on the vein so that a 1 or 2 inch length of vein lies between the thumb and the tourniquet. As soon as the vein is entered, the thumb is removed.

f. Immersing the part in warm water or applying hot compresses to the area will cause veins to fill in some difficult cases.

## OBTAINING CLEAR SERUM

1. Draw before meals to avoid presence of chyle which appears in serum after a fatty meal.

2. Use sterile needles and glassware.

3. Remove the needle and run the blood gently down the side of the tube to avoid rupture of cells.

4. Let stand at room temperature undisturbed for at least 30 minutes to clot.

5. Keep specimens in refrigerator until time for examination. Freezing will cause hemolysis.

6. If blood or serum is to be mailed:

   a. Collect under sterile conditions.

   b. Keep specimen and packing material in refrigerator until time for mailing.

   c. Mail the same day as it is collected because serum possesses antibacterial qualities only when fresh.

   d. If serum is to be mailed, separate from the clot under sterile conditions.

   e. Do not heat serum before mailing. Heat destroys its antibacterial properties.

   f. For further instructions regarding the shipment of serums for routine serologic testing see Section X, page 101.

## SECTION III

## PREPARATION OF GLASSWARE

There are various ways of cleaning glassware. An effective procedure will be outlined. Since traces of acids and/or alkali alter results with the precipitation and agglutination tests, and are also toxic to complement, it is imperative that glassware be free of these substances. The newer detergents (such as Dreft and Vel) require multiple rinses as traces sufficient to hemolyze erythrocytes can be present after seventeen rinses.

Pipets: 1.0, 5.0, 10.0 ml.

1. Place pipets in soapy water, points up, for one hour.

2. Rinse pipets in an automatic rinser for 15 minutes. NOTE: Adjust the flow so that the washer alternately fills and empties. If the flow of water is too slow, the washer fails to empty; if too fast, it will not fill.

3. Drain the pipets, dry in a hot air oven, and permit to cool.

4. Place in dichromate cleaning solution, preferably overnight. Drain off the bulk of the cleaning solution.

5. Place in the automatic pipet washer taking care to remove any cleaning solution that has dripped onto the top of the washer or inside the washer at levels above the mark to which the water automatically rises. Such traces of cleaning solution will contaminate the effects of rinsing.

6. Permit the pipets to rinse for an hour, checking to see that the washer is alternately filling and emptying.

7. Place the pipets in distilled water for thirty minutes.

8. Drain and dry in the oven.

NOTE: The inside of the pipet washer should be scrubbed and rinsed periodically.

Cleaning Solution Formula (Dichromic Acid)

| | |
|---|---|
| Concentrated sulfuric acid ..... | 460 gm. (252 ml.) |
| Water ..... | 300 ml. |
| Sodium or potassium dichromate ..... | 60 gm. |

Procedure

1. Mix water and $Na_2Cr_2O_7$ until dissolved.

2. Add $H_2SO_4$ very slowly, stirring constantly until $Na_2Cr_2O_7$ is reprecipitated.

Great caution must be taken in adding the sulfuric acid to the sodium dichromate solution. The solution becomes intensely hot and may boil over. The transfer of cleaning solution from one jar to another may be carried out by a siphon arrangement as follows: A rubber tube is attached to the suction arm of a water vacuum pump connected with a faucet. The far end of the rubber tube is lowered to the bottom of the jar that is to be emptied of cleaning solution. By starting a stream of water through the faucet, a vacuum is created within the rubber tubing and the cleaning solution is at once drawn up into the tubing. By detaching this tubing from the faucet and placing it in another jar, the acid will continue to flow into that jar. A thin acid-resistant rubber tubing is employed for this purpose, making it possible to see readily the cleaning solution rising within the tube.

Pipets: 0.1 and 0.25 ml.

These pipets have a very small bore rendering the mechanical washer ineffective. Therefore, these soiled pipets should be placed in a cylinder of water. They should be cleaned by pulling tap water through them with the aid of a water suction pump, followed by distilled water. For rapid drying, one may follow the distilled water with a rinse of alcohol and then ether, still employing the vacuum pump to pull the fluids through the pipet.

Procedure for washing tubes

Clot tubes. Clots should be emptied into a wide-mouthed drain followed by copious amounts of cold water. Tubes should be rinsed with water and placed in soap and water solution.

Kahn tubes and other tubes except Colloidal Gold tubes

1. Empty contents of tubes in the sink.

2. Submerge tubes in a bucket of soapy water.

3. Wash each tube individually with a brush.

4. Rinse tubes 5 times with running cold water.

5. Rinse tubes 2 times in distilled water. (Two buckets of distilled water may be used. Rinse in Bucket I and then Bucket II. Change the distilled water at least every day and more often if the volume of glassware is great.)

Colloidal Gold tubes

1. Empty contents of tubes in sink.

2. Scrub tubes with soapy water. Use a brush and scrub each tube individually.

3. Tubes are rinsed 3 times with tap water and 2 times with distilled water.

4. Tubes are dried and then placed in aqua regia overnight. (For formula see Section IX on spinal fluid.)

5. Remove tubes from aqua regia.

6. Each tube is rinsed directly under a stream of running tap water. (Hold a few tubes in the hand and rinse under the faucet.) Fill and empty tubes ten times, and as the tube is inverted rinse the outside.

7. Rinse each tube individually three times with distilled water by holding the tubes under distilled water flowing from a bottle.

NOTE: All buckets used should be scrubbed, thoroughly rinsed, and turned over to drain at the end of each working day.

## SECTION IV

## KAHN TEST PROCEDURES

The techniques given in the following pages include the standard diagnostic test and quantitative test with blood and spinal fluid and the presumptive test with blood.

### Apparatus

The use of standard apparatus in the performance of the various procedures of the Kahn tests is absolutely essential for correct results.

1. Kahn test tubes: These are used for the performance of tests with serum and spinal fluid. They are 75 mm. in length and 10 mm. in inside diameter, 12 mm. outside diameter, and are made of Pyrex glass.

2. Antigen suspension vials: These vials, with straight wall and flat bottom used for preparing Kahn antigen suspensions, are 55 mm. in length and 15 mm. in inside diameter.

3. Pipets: The following pipets are needed:

   5.0 ml. graduated to 0.1 ml.
   1.0 ml. graduated to 0.01 ml.
   0.25 ml. graduated to 0.0125 ml. (for pipetting antigen suspension).
   0.2 ml. graduated to 0.001 ml.

4. Blood specimen test tubes: The recommended size of Pyrex test tubes for the collection of blood specimens is 100 mm. by 15 mm.

5. Serum test tubes: The recommended size of Pyrex test tubes for holding the individual serums to be tested is 100 mm. by 13 mm., outside diameter.

6. Kahn test tube rack: This is made of suitable material (preferably rubberized sheet copper), is 3 inches wide, 11 1/2 inches long, 2 3/4 inches high, and consists of three shelves, the upper and middle ones containing three rows of holes, each approximately half an inch in diameter. The center row of holes is offset half an inch to the left.

7. Kahn shaking apparatus: This apparatus should have a speed of 275 to 285 oscillations per minute,* with a stroke of 1 1/2 inches.

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* Generally attained when operated with a motor on 115 volt, 60 cycles A.C. line under a load ranging from 5-11 pounds.

8. Water bath: It must be of such type as to maintain a temperature of 56°C.

9. Centrifuges and metal centrifuge tubes.

## Reagents

### Standard Kahn Antigen

This antigen is employed in the performance of the standard (diagnostic) tests with serum and spinal fluid, and in the quantitative tests with serum and spinal fluid.

Kahn standard antigen has been standardized to a degree of specificity and sensitivity required for standard Kahn tests. The bringing of each lot of antigen to the required standard is of first importance for correct results, and each lot of Kahn antigen manufactured and standardized at the United States Naval Medical School is checked and rechecked by Dr. Kahn's laboratory. The titer or number of ml. of saline to use with 1 ml. of antigen is given on the label.

### Care of Standard Antigen

1. Only chemically clean and dry glass vessels should be used for storing antigen and these should be tightly stoppered, because evaporation of alcohol from the antigen will not only change the titer of the antigen but may cause separation of the cholesterol.

2. Antigen should be kept at room temperature - never in the refrigerator nor in the incubator. It should be stored in the dark, as in a cupboard. The antigen bottle in daily use might be kept in a mailing container to avoid undue exposure to light.

3. Antigen should not come into contact with rubber or cork because both contain alcohol-soluble elements which affect antigen titer and specificity. Such a stopper should be covered with thin, high-grade tinfoil or an approved substitute.

4. Changes in antigen due to aging are generally reflected in the appearance of nonreactive test results. If these reactions become too sharply clear or if they become cloudy, the antigen should be retitrated and restandardized. The need for retitration and/or restandardization of this reagent occurs but rarely. Such procedures should be carried out only by a laboratory qualified to make adjustments.

5. If there is the slightest indication that a given lot of standard antigen has undergone change, it should be returned to the United States Naval Medical School with a full statement of the facts.

### Serum

1. It is essential that the serum employed in the test be entirely free from cells

or particles of any kind, since these may give the impression of a precipitate in the completed test.

2. It is important to adhere to sterile technique in obtaining blood from patients. The tube into which the blood is emptied must be chemically clean and dry. Sterility of the tube is desirable but not essential, except when the specimen is to be shipped and several days intervene before examination. If the tube is agitated before a clot is formed, there will be a tendency toward hemolysis. After a clot is formed, this tendency is greatly reduced.

3. It is well to separate the blood clot with wooden applicators before centrifugation. The inner wall of the tube is encircled with the applicators so as to separate completely the adhering blood clot. If the clot is contracted, it may be removed before centrifugation. The same applicators should never be used for more than one specimen of blood.

4. Centrifugation for 10 to 15 minutes at 2,000 revolutions per minute is usually sufficient for the separation of the serum from the clot. If serum is not absolutely clear, it should be recentrifuged.

5. The clear supernatant serum either is poured off into a clean tube or is pipetted off with a serum-transfer, bulb-capillary pipet. The same pipet should never be used for more than one specimen until it is recleaned thoroughly.

6. The clear serum should be heated (inactivated) for 30 minutes in a water bath at a temperature of 56°C. before performing the test.

7. Serums should be tested as soon as possible after being heated. For uniformity, it is well to begin the performance of the tests within about 10 minutes after the serums have been removed from the 56°C. water bath. Serums that have been heated 2 to 24 hours previously should be reheated for 10 minutes at 56°C. when they are to be re-examined; if after 24 hours, they should be reheated for 15 minutes.

8. Serums showing some hemolysis or containing chyle or bile do not affect the correctness of Kahn results. But if markedly hemolyzed or decomposed because of bacterial contamination, they are not fit for serologic tests.

Physiologic Saline Solution for Kahn Test

This saline solution consists of 0.9 per cent sodium chloride (reagent quality for biological work) in distilled water. This sodium chloride must be chemically pure and of reagent grade. Sodium chloride of inferior quality (such as salt tablets) must not be used in the Kahn tests. Aboard ship, C. P. sodium chloride tablets for intravenous use may be used if no other C. P. sodium chloride is available.

## Standard Kahn Procedures

### Standard (Diagnostic) Test with Serum

The standard test is a three-tube test, each tube containing a different proportion of serum to antigen suspension. Optimum precipitation is obtained when the concentration of antigen and antibody (reagin) approximate one another. Hence, a relatively large, moderate, and small quantity of antigen suspension is employed with each serum, since the serum may contain a large, moderate, or small amount of antibody. Then again, the use of three proportions of serum to antigen suspension makes it possible to obtain highly sensitive precipitation results with standard antigen, which is an antigen of moderate sensitivity but high specificity.

### Preliminary Preparations for Test

Proper coordination and sequence of the various steps of the test, in relation to the number of specimens to be examined, are important for maintaining a high level of accuracy and efficiency. The racks should be set up, tubes numbered, and pipets ready for measuring antigen suspension and serum. Antigen suspension is prepared as needed; hence, the amount prepared must conform to the number of tests to be made. It is always best for the technician to perform Kahn tests alone, so the number of tests to be performed at any one given time will depend upon how many tests the technician can accurately finish in the prescribed 20-minute period. It is usually not advisable to perform over 40 standard tests at one time.

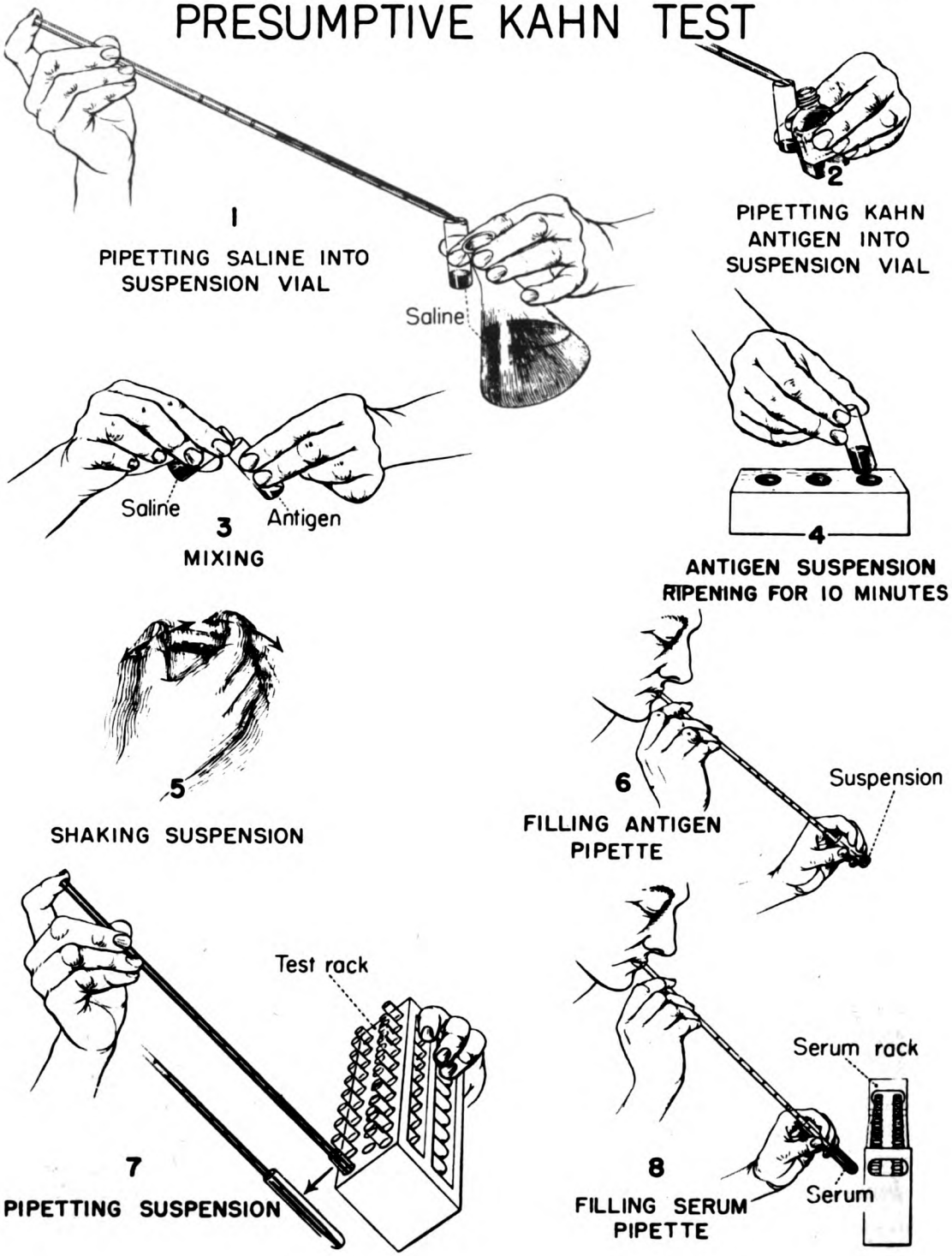
### Heating of Serum

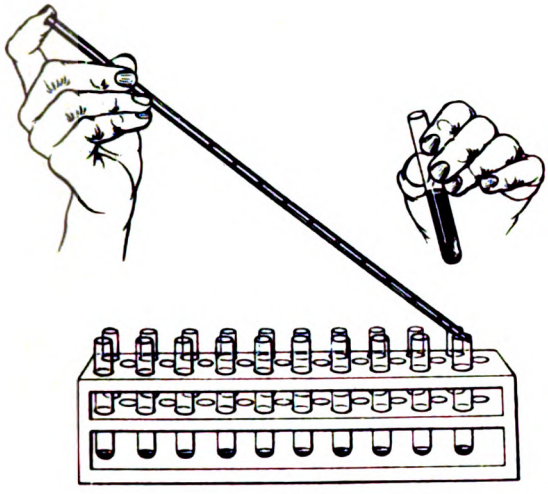
The serum, after it has been heated for 30 minutes at 56°C., is examined for the presence of particles. If present, the serum is cleared by recentrifuging. (Occasionally a serum will show the presence of particles after heating, although it was entirely clear before heating.)

### Preparation of Standard Antigen Suspension

This suspension is prepared shortly before the serums are taken from a 56°C. water bath. Antigen is mixed with saline solution according to the required titer. Thus, if the titer is 1 ml. antigen plus 1.2 ml. 0.9 per cent saline solution, the antigen is mixed as follows: (1) 1.2 ml. saline solution is measured (with a 1.0 ml. or 2.0 ml. pipet) into a chemically clean and dry standard antigen suspension vial; (2) 1 ml. antigen is measured (with a chemically clean and dry 1.0 ml. pipet) into a similar vial; (3) the saline solution is poured into the antigen, and as rapidly as possible without waiting to drain the vial, the mixture is poured back and forth a total of 12 times to insure thorough mixing; (4) the antigen suspension is allowed to stand for 10 minutes before using. The suspension is not to be used after standing over 30 minutes from the time of mixing. A previously prepared antigen suspension

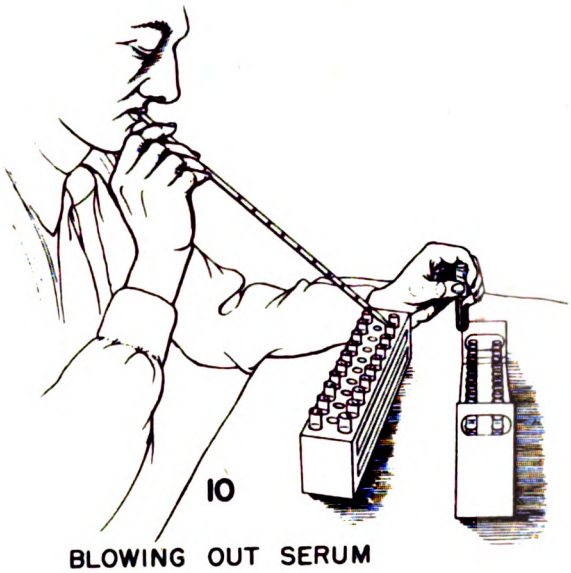
# BASIC STEPS IN ALL KAHN TEST PROCEDURES AS ILLUSTRATED BY THE PRESUMPTIVE KAHN TEST





9

PIPETTING SERUM INTO ANTIGEN SUSPENSION

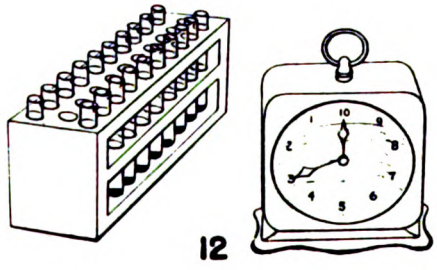


10

BLOWING OUT SERUM

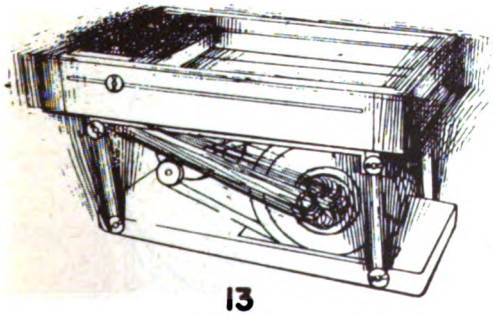
11

SHAKING FOR 10 SECONDS



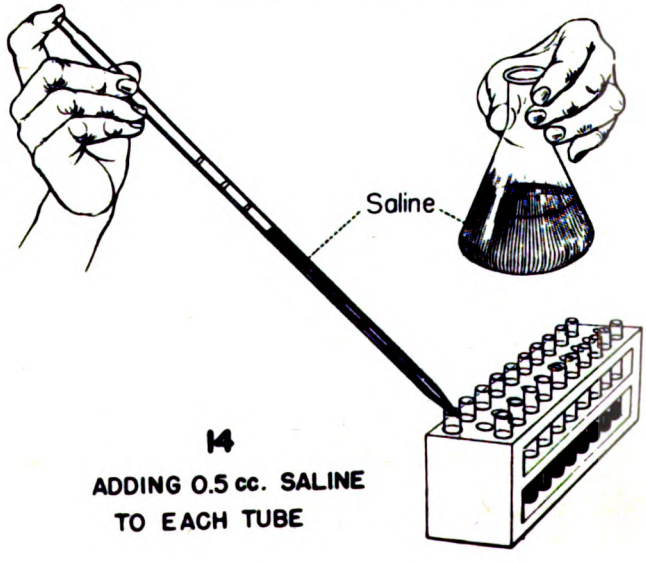
12

TESTS STANDING FOR 3 MINUTES



13

SHAKING FOR 3 MINUTES

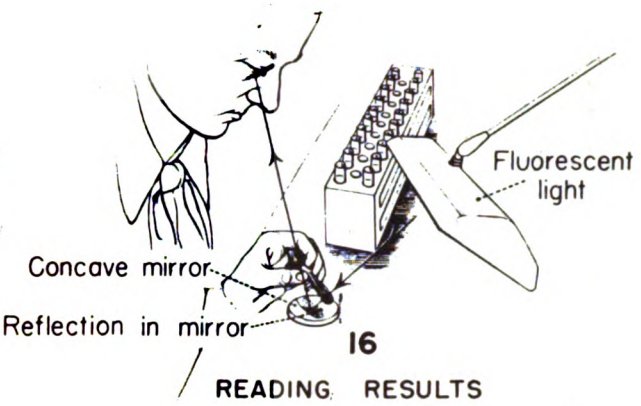



14

ADDING 0.5 cc. SALINE TO EACH TUBE

15

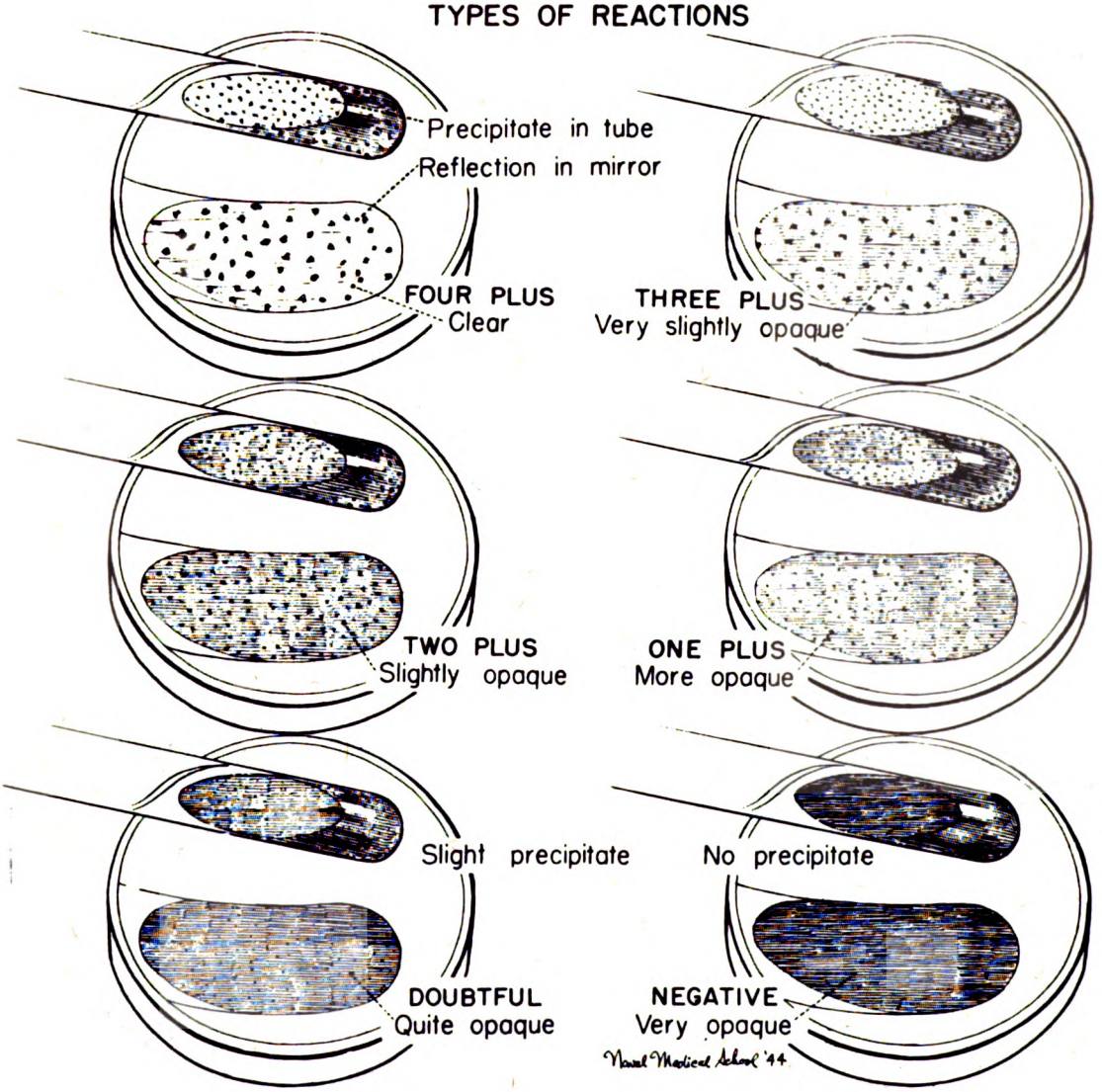
**SHAKING TESTS**
(To mix ingredients)




16

**READING RESULTS**

## TYPES OF REACTIONS



is not to be mixed with a newly prepared suspension. More than 1 ml. of antigen may be mixed with a proportionately larger amount of saline solution. Thus, in the case of an antigen with the above titer, 2 ml. may be mixed with 2.4 ml. saline solution or 2.5 ml. with 3.0 ml. saline solution. Amounts of antigen less than 1 ml. or more than 3.0 ml. for the preparation of an antigen suspension should not be used.

Measuring Antigen Suspension

The antigen suspension is shaken well, and 0.05 ml., 0.025 ml., and 0.0125 ml. amounts are measured for each serum to be tested, delivering the suspension to the bottom of the tubes. The standard rack capacity is 30 tubes; 0.05 ml. amounts are measured into the tubes of the first row, 0.025 ml. amounts into the tubes of the second row, and 0.0125 ml. amounts into the tubes of the third row. (If the antigen suspension pipet is broken at the tip, it will not deliver these amounts accurately.) Pipet antigen suspension into one rack at a time, to be followed at once by the serum before pipetting the suspension into another rack.

Measuring Serum

The serum is added as soon as possible after the antigen suspension has been pipetted to avoid undue evaporation of the suspension. It is of the utmost importance to use a clean, dry pipet for each serum. Each serum, in 0.15 ml. amounts, is added to the 0.05 ml., 0.025 ml., and 0.0125 ml. amounts of antigen suspension, and the rack of tubes is shaken vigorously for 10 seconds to insure thorough mixing of the ingredients. Permit serum-antigen suspension mixture to stand for 3 to 7 minutes at room temperature before the mechanical shaking for 3 minutes. (See Shaking below.)

Controls

Before measuring the antigen suspension and serum for the regular test, three control tests are carried out, one with a positive serum, one with a negative serum, and one employing saline solution instead of serum. The antigen suspension for these controls is pipetted immediately after it has stood for 10 minutes. After pipetting the serums (and saline), the tests are at once shaken for 3 minutes. This control setup, completed in not more than 5 minutes, permits the reading of the results of the control tests before performing the regular tests and makes possible the detection of any error in the preparation of the antigen suspension.

Shaking

The tests are shaken in a Kahn standard shaking machine for 3 minutes.

Addition of Saline Solution

After the 3 minute shaking period, remove the rack from the shaking machine. Add 1.0 ml. of saline solution to each tube of the front row (containing 0.05 ml. of antigen emulsion) and 0.5 ml. amounts to all other tubes. NOTE: Add saline to one rack and complete readings before adding saline solution to another rack. Shake the rack gently by hand for a few seconds to mix the contents of tubes.

Time of Reading Results

Read each tube of the rack within 2 minutes after the addition of saline solution. (See Reading and Reporting of Results). Reread each tube 15 minutes after the first reading was taken and in all instances where a negative reading was not obtained on the first reading. Record the results of the first and second readings of each tube.

Table 1A. Summary of Standard Kahn Procedure

| | Tube 1 (front) | Tube 2 (middle) | Tube 3 (back) |
|---|---|---|---|
| Serum-antigen suspension ratios | 3:1 | 6:1 | 12:1 |
| Antigen suspension, ml. | 0.05 | 0.025 | 0.0125 |
| Quantity of serum, ml. | 0.15 | 0.15 | 0.15 |
| Shake (by hand) 10 seconds. | | | |
| Allow to stand 3 minutes. | | | |
| Shake (Kahn shaker) 3 minutes. | | | |
| Quantity of saline added, ml. | 1.0 | 0.5 | 0.5 |
| Shake gently by hand. | | | |
| Make first reading of results. | | | |
| Re-examine 15 minutes later. | | | |

## Results of Standard Test

Method of Reading Results

1. Place a microscope mirror on the work bench with the concave side upward.

2. Adjust a reading lamp (daylight bulb or fluorescent tube) 3 to 6 inches above the mirror so that the bulb image is not visible, but so that the tube can be held in the cone of light. (It is best to have only one source of light during the reading, and the surface of the work bench should not reflect light.)

3. Place each tube to be read in a nearly horizontal position with the lower portion of the tube about 1 to 2 inches above the mirror.

4. View the image of the tube contents in the mirror and note the degree of flocculation.

5. Results may also be read by window light or by slit-lamp. (Kahn, R. L.: Serology with Lipid Antigens; Williams and Wilkins, Baltimore, 1950.)

6. For uniform results, it is important for an individual reader to adhere to one method of reading flocculation results.

Recording Results

A permanent record should be made of findings in all tubes of each test at the time of reading. All tests should be read independently by two workers. When two workers are not available, the original reading should be checked by the same worker after an interval of 15 minutes.

Record degrees of flocculation in accordance with the following scale:

4+ ..... Relatively large floccules suspended in a clear medium.

3+ ..... Medium-sized floccules suspended in a clear or very slightly cloudy medium.

2+ ..... Fine floccules easily distinguishable in a somewhat cloudy medium.

1+ ..... Very fine floccules distinguishable in a somewhat cloudy medium.

± ..... Extremely fine floccules just distinguishable in a somewhat cloudy medium.

Negative ..... An opalescent medium free from from visible particles. (Non-reactive)

Interpreting Results

1. Strongly potent serums show complete or four plus flocculation in each of the three tubes, but, because of the different amounts of antigen suspension employed, the floccules are unequal in bulk; they are greatest in the first tube and least in the last tube.

2. Serums that are not strongly potent do not show complete flocculation in each of the three tubes. Such serums show most marked flocculation in the third tube because the small amount of antibody (reagin) reacts best with a small amount of antigen

suspension. These serums generally show weak flocculation in the middle tube, which contains a moderate amount of antigen suspension, and no flocculation in the first tube, which contains a relatively large amount of the suspension.

3. Another type of flocculation reaction is met with occasionally, namely, one in which flocculation is marked in the first tube and weak or negative in the second or third tube. In this instance, the serum generally is so markedly rich in antibody that it requires a relative excess of antigen suspension to give maximum flocculation. When a reaction of this type is encountered, it is necessary to set up a supplementary test in which the amount of antigen suspension in relation to serum is increased beyond that employed in the standard test.

## Reporting Results

1. In all reactions where the greatest degree of flocculation is produced by the lesser amounts of antigen suspension (tubes 2 and 3), the following scheme of reporting should be used:

Table 1 B. Reporting Results

| Sum of pluses in six-tube readings | Report | New Method of Reporting |
|---|---|---|
| 22 to 24 | Positive (4+) | Reactive (4+) |
| 16 to 21 | Positive (3+) | Reactive (3+) |
| 10 to 15 | Positive (2+) | Reactive (2+) |
| 5 to 9 | Doubtful (1+) | Weakly Reactive (1+) |
| 4* | Doubtful (±) | Weakly Reactive (±) |
| 3 or fewer | Negative | Non-Reactive |

± Readings are disregarded.

*Exceptions: A sum of 4 pluses should be given a Nonreactive report when the results are - 1 1 and - 1 1 on first and second readings.

2. The manner of reporting other types of reactions is described in the following tables:

Table 2. Zonal reactions reported "Reactive (4+)" (supplementary tests not necessary)

| Serum No. | First reading | | | Second reading | | |
|---|---|---|---|---|---|---|
| | Tube 1 | Tube 2 | Tube 3 | Tube 1 | Tube 2 | Tube 3 |
| 1 | 4 | 4 | 3 | 4 | 4 | 3 |
| 2 | 4 | 4 | 2 | 4 | 4 | 2 |
| 3 | 4 | 4 | 1 | 4 | 4 | 1 |
| 4 | 4 | 4 | — | 4 | 4 | — |

**Table 3. Zonal reactions reported "Reactive (4+)" provided supplementary tests 1 and 2 are Reactive**

| Serum No. | First reading | | | Second reading | | |
|---|---|---|---|---|---|---|
| | Tube 1 | Tube 2 | Tube 3 | Tube 1 | Tube 2 | Tube 3 |
| 1 | 4 | 3 | 3 | 3 | 3 | 3 |
| 2 | 4 | 3 | 2 | 4 | 3 | 2 |
| 3 | 4 | 3 | — | 4 | 3 | — |
| 4 | 4 | 1 | — | 4 | — | — |
| 5 | 3 | 3 | 3 | 3 | 3 | 3 |
| 6 | 2 | 2 | 2 | 2 | 2 | 2 |
| 7 | 2 | — | — | 2 | — | — |
| 8 | 1 | 1 | 1 | 1 | 1 | 1 |
| 9 | 1 | — | — | 1 | — | — |
| 10 | ± | ± | ± | ± | ± | ± |
| 11 | ± | — | — | ± | — | — |

**Table 4. Zonal reactions reported "Reactive (3+)" provided supplementary tests 1 and 2 are Nonreactive**

| Serum No. | First reading | | | Second reading | | |
|---|---|---|---|---|---|---|
| | Tube 1 | Tube 2 | Tube 3 | Tube 1 | Tube 2 | Tube 3 |
| 1 | 4 | 3 | 3 | 3 | 3 | 3 |
| 2 | 4 | 3 | 2 | 4 | 3 | 2 |
| 3 | 3 | 3 | 3 | 3 | 3 | 3 |

**Table 5. Zonal reactions reported "Reactive (2+)" provided supplementary tests 1 and 2 are Nonreactive**

| Serum No. | First reading | | | Second reading | | |
|---|---|---|---|---|---|---|
| | Tube 1 | Tube 2 | Tube 3 | Tube 1 | Tube 2 | Tube 3 |
| 1 | 4 | 2 | 2 | 3 | 2 | 2 |
| 2 | 4 | 1 | 1 | 4 | 1 | 1 |
| 3 | 4 | 3 | ± | 4 | 3 | ± |
| 4 | 3 | 3 | 2 | 3 | 3 | — |

**Table 6. Zonal reactions reported "Weakly Reactive (1+)" provided supplementary tests 1 and 2 are Nonreactive**

| Serum No. | First reading | | | Second reading | | |
|---|---|---|---|---|---|---|
| | Tube 1 | Tube 2 | Tube 3 | Tube 1 | Tube 2 | Tube 3 |
| 1 | 4 | 1 | ± | 3 | 1 | ± |
| 2 | 3 | 2 | ± | 3 | — | — |
| 3 | 3 | ± | — | 3 | ± | — |
| 4 | 2 | 2 | 2 | 2 | 2 | 2 |

**Table 7. Zonal reactions reported "Weakly Reactive (±)" provided supplementary tests 1 and 2 are Nonreactive**

| Serum No. | First reading | | | Second reading | | |
|---|---|---|---|---|---|---|
| | Tube 1 | Tube 2 | Tube 3 | Tube 1 | Tube 2 | Tube 3 |
| 1 | 2 | 2 | 1 | 2 | 2 | ± |
| 2 | 2 | 2 | — | 2 | 1 | — |
| 3 | 2 | 1 | — | 2 | ± | — |

**Table 8. Zonal reactions reported "Nonreactive" provided supplementary tests 1 and 2 are Nonreactive**

| Serum No. | First reading | | | Second reading | | |
|---|---|---|---|---|---|---|
| | Tube 1 | Tube 2 | Tube 3 | Tube 1 | Tube 2 | Tube 3 |
| 1 | 2 | ± | ± | 2 | ± | ± |
| 2 | 2 | ± | ± | 2 | — | — |
| 3 | 2 | ± | — | 2 | — | — |
| 4 | 2 | — | — | 2 | — | — |
| 5 | 1 | 1 | ± | 1 | ± | ± |
| 6 | 1 | ± | ± | 1 | — | — |
| 7 | ± | ± | ± | ± | — | — |
| 8 | ± | — | — | ± | — | — |

## Supplementary Kahn Tests with Serum

### Supplementary Test No. 1

| | Tube 1 | Tube 2 |
|---|---|---|
| Ratio (Serum-Antigen) | 1:1 | 2:1 |
| Antigen suspension, ml. | 0.05 | 0.05 |
| Serum, ml. | 0.05 | 0.1 |

1. Pipet 0.05 ml. of antigen suspension to the bottom of each of two tubes (numbered 1 and 2).

2. Add 0.05 ml. of serum (previously heated) to tube 1 and 0.1 ml. of serum to tube 2.

3. Shake rack by hand for 10 seconds to mix contents of tubes. Allow to stand three to seven minutes.

4. Shake rack on Kahn shaker for 3 minutes.

5. Remove rack from shaker, add 1.0 ml. of saline solution to each tube, and shake by hand to mix.

6. Read immediately, and again after 15 minutes, as described under "Reading of Results."

Reactive (Positive) ..... A 4+ or a 3+ reaction in each of the two tubes.
Nonreactive (Negative) .... Less than a 3+ reaction in each of the two tubes.

### Supplementary Test No. 2

1. Prepare serum dilutions 1:4, 1:8, and 1:16 in the following manner:

2. Pipet into three tubes (numbered 1, 2, and 3) 0.6, 0.4, and 0.4 ml. of saline solution, respectively.

3. Add 0.2 ml. of serum to tube 1 and mix.

4. Transfer 0.4 ml. from tube 1 to tube 2 and mix.

5. Transfer 0.4 ml. from tube 2 to tube 3 and mix.

6. Pipet 0.0125 ml. of antigen suspension (see Kahn Standard Test) to the bottom of three Kahn tubes (numbered 1, 2, and 3).

7. Add 0.15 ml. of the 1:4, 1:8, 1:16 dilutions of serum to tubes 1, 2, and 3, respectively, starting with the highest serum dilution.

8. Shake rack by hand 10 seconds to mix the contents of tubes. Allow to stand 3 to 7 minutes.

9. Shake rack on the Kahn shaker for 3 minutes.

10. Remove rack from the shaker, add 0.5 ml. of saline solution to each tube, and shake to mix.

Read immediately as described under "Reading of Results."

Reactive (Positive) ..... A 4+ or 3+ reaction in at least the 1:4 dilution.

Nonreactive (Negative) .. A 2+ reaction or less, in the 1:4 and higher dilutions.

Reporting of Results

1. Supplementary tests 1 and 2 Reactive (positive).
   Report results of the standard test as Reactive (positive) 4+.

2. Supplementary tests 1 and 2 Nonreactive (negative).
   Report results of the standard test as indicated in tables 4 through 8.

3. Supplementary test 1 Reactive and supplementary test 2 Nonreactive.
   Prepare 1:32, 1:64, and 1:128 dilutions of the serum, and test as in supplementary test 2. If the result is Reactive in one or more tubes (a 4+ or 3+ reaction), report the results of the standard test as Reactive (positive) 4+. If the results are Nonreactive (negative) in all tubes (less than a 3+ reaction), report the results of the standard test as indicated in tables 4 through 8.

4. Supplementary test 1 Nonreactive (negative) and supplementary test 2 Reactive.
   Test the undiluted serum in a 1:2 ratio of serum: antigen suspension (0.05 ml. of antigen suspension plus 0.025 ml. of serum). If the result is reactive (a 4+ or a 3+ reaction), report the results of the standard test as Reactive (positive) 4+. If the results are Nonreactive or negative (less than 3+ reaction), report the results of the standard test as indicated in tables 4 through 8.

## Quantitative Kahn Test with Serum

The standard test is essentially qualitative in nature. When the result is Reactive 4+, no indication is obtained as to the quantitative potency of the serum. Two serums giving positive reactions may differ markedly in their potency. The extent of this difference may be readily determined by means of the quantitative test with serum. The test consists of two steps. Reactive 4+ or 3+ serums are first diluted in series with saline solution. Each serum dilution is then examined with standard antigen suspension. The highest dilution giving definite flocculation (4+, 3+, or 2+) is the end point desired. The apparatus and reagents are the same as for the standard test. The quantitative test should be performed on all serum specimens producing three plus or four plus reactions in the standard Kahn test as well as those specimens giving reverse reactions.

### Dilution of Positive (Reactive) Serum with Saline Solution

Ten Kahn tubes are set up. The accompanying outline gives the scheme for preparing the serial dilutions of serum. (Use a 1 ml. or 2 ml. pipet.)

Table 9. Serum Dilution

| Tube No. | Saline 0.9 per cent | Dilution procedure | Dilutions | Kahn units |
|---|---|---|---|---|
| 1 | 0.3 ml. plus | 0.3 ml. of undiluted serum | 1:2 | 8 |
| 2 | 0.3 ml. plus | 0.3 ml. of dilution from tube No. 1 | 1:4 | 16 |
| 3 | " | 0.3 ml. of dilution from tube No. 2 | 1:8 | 32 |
| 4 | " | 0.3 ml. of dilution from tube No. 3 | 1:16 | 64 |
| 5 | " | 0.3 ml. of dilution from tube No. 4 | 1:32 | 128 |
| 6 | " | 0.3 ml. of dilution from tube No. 5 | 1:64 | 256 |
| 7 | " | 0.3 ml. of dilution from tube No. 6 | 1:128 | 512 |
| 8 | " | 0.3 ml. of dilution from tube No. 7 | 1:256 | 1,024 |
| 9 | " | 0.3 ml. of dilution from tube No. 8 | 1:512 | 2,048 |
| 10 | " | 0.3 ml. of dilution from tube No. 9 | 1:1,024 | 4,096 |

Note: The serum dilutions should not be allowed to stand longer than 30 minutes before their transfer to the antigen suspension tubes. Preferably, the serum dilutions should be employed within 20 minutes after their preparation.

### Performance of Test

The antigen suspension is prepared in the usual manner by mixing Kahn standard antigen with 0.9 per cent saline solution, according to the antigen titer. After standing for 10 minutes (but not longer than 30 minutes) antigen suspension is pipetted in 0.0125 ml. amounts into each of ten Kahn test tubes, depositing the suspension at the bottom of the tubes. With the pipet that has remained standing in the highest serum dilution

(tube 10), 0.15 ml. is transferred into the corresponding tube 10 containing the antigen suspension. Then, with the same pipet, 0.15 ml. of serum dilution (tube 9) is transferred to antigen suspension tube 9. In the same way, all the serum dilutions are transferred to the antigen suspension tubes in series. Shake the rack by hand for 10 seconds, allow to stand 3 to 7 minutes, shake on a mechanical shaker for 3 minutes. Remove rack from the shaker and add 0.5 ml. of saline to each tube. Add saline to one rack and complete readings before adding saline to another rack. The rack is shaken by hand for a few seconds to mix contents of the tubes. Read each tube of the rack within 2 minutes after the addition of saline.

Determination of Kahn Units

A definite precipitate (4, 3, or 2+) is recorded as positive, while a very weak (+ or ±) reaction is discounted. If a serum gives a 4, or 3+ reaction (in an undiluted state) and is negative in the dilution series, it is considered for simplicity in calculation as containing four or three units respectively. The potency of any serum which is positive on dilution is determined according to the formula S equals 4D, where S is the serum potency in terms of Kahn units and D is the highest dilution ratio giving a positive (4, 3, or 2+) reaction. Thus if the serum dilution 1:4 is positive and 1:8 and higher dilutions are negative, the serum contains 4 times 4 or 16 Kahn units. If the serum dilution 1:8 is positive and 1:16 and higher dilutions are negative, the serum contains 32 units. Report both in Kahn units and the highest dilution giving a 2+ or more reaction. Example: 256 Kahn units (1:64 dilution).

Standard (Diagnostic) Test with Spinal Fluid

See Section on Cerebrospinal Fluids.

Quantitative Test with Spinal Fluid

See Section on Cerebrospinal Fluids, section on Quantitative Kahn Test.

Presumptive Kahn Test with Serum

The presumptive test is an auxiliary method to the standard Kahn test. The main difference between the two methods is that the presumptive is more sensitive than the standard test. This increased sensitivity is due to the fact that, in the presumptive test, sensitized antigen is employed which is more sensitive than standard antigen. A negative reaction with this test is "presumptive" evidence that syphilitic reagin is not present and the conclusion that syphilis is not present in the patient can be relied upon with an extremely small chance of error. The presumptive test is of value (1) as a technical check on the standard test, (2) in treated cases of syphilis when a highly sensitive method may be desired, and (3) as an additional criterion in

establishing the absence of syphilis. The presumptive test is an excellent "screen" test and is used as such in the Department of Serology at the United States Naval Medical School. All specimens are examined with the presumptive Kahn test and if they are negative, they are reported without further examination. A negative reaction by the Kahn presumptive test is more reassuring since the Kahn presumptive test is more sensitive than the Kahn standard test. Every serum that shows any reaction whatsoever by the Kahn presumptive test must be checked by the Kahn standard (diagnostic) test. Note: State laws demand that premarital tests be standard tests. For these a standard Kahn must be run.

Statements under Standard Kahn Antigen (1, 2, 3, and 4) on page 15 apply also to sensitized antigen.

Arrange Kahn test tubes in a rack so that there is one tube for each serum to be tested, including positive serum, negative serum and saline controls. Number tubes to correspond with the serums being tested.

Preparation of Antigen Suspension

1. Measure into an antigen suspension vial the proper amount of saline required by the titer to prepare sensitized antigen suspension.

2. Measure 1.0 ml. sensitized antigen into another vial.

3. Pour the saline solution into the antigen and as rapidly as possible pour the mixture back and forth 12 times without waiting to drain the vials.

4. Allow antigen suspension to stand 10 minutes before using. (Discard antigen suspension after it has aged more than 30 minutes.)

5. Place thumb over the mouth of the mixing vial and shake briskly to suspend antigen particles evenly.

6. Pipet 0.025 ml. of sensitized antigen suspension directly to the bottom of each tube.

7. Add 0.15 ml. of heated serum to each corresponding tube.
Note: Complete the addition of sensitized antigen suspension and serum to one rack before adding sensitized suspension to another.

8. Shake rack by hand for ten seconds.

9. Allow rack to stand 3 to 7 minutes at room temperature.

10. Shake rack on the Kahn shaker for 3 minutes.

11. Remove rack from the Kahn shaker and add 0.5 ml. of saline to each tube.
Note: Add saline to one rack and complete reading before adding saline to another.

12. Read each tube of the rack as described under "Reading of Results" immediately after the saline has been added.

Interpretation of Results

The results are read in the same manner as the standard Kahn test. Complete precipitation, four or three plus, is interpreted as positive, a moderate precipitation reaction, two plus, is interpreted as doubtful, and tubes showing 1+ or ± as negative.

Serum Control

Examine each serum for foreign particles which might give the appearance of a specific precipitate. Particularly in the case of each positive reaction, it is essential to determine that the serum used in the test is free from foreign particles. In using the presumptive test as a check on the standard Kahn test, the same serum control is, of course, sufficient for both methods.

Note: In performing the presumptive test, it is important to watch the appearance of the negative reactions. These reactions should be neither "cloudier" nor "clearer" than the negative reactions of the standard test. If the negative reactions of the presumptive test approach water clarity, positive reactions are likely to be missed; if the reactions are cloudy, many nonspecific, weak reactions may occur. When an undue number of doubtful or weakly positive reactions are obtained, negative with the standard test, the indications are that the presumptive test gives "cloudy" negatives. This situation can be readily overcome by increasing the saline titer generally by 0.05 ml., rarely by 0.1 ml. If the negative reactions are too clear, the amount of saline in the titer may be decreased also by 0.05 ml., rarely by 0.1 ml.

Effect of Temperature on the Kahn Tests.

The Kahn procedures should be performed at room temperature. Kahn tests should never be performed in a cold room, or with cold reagents. When Kahn tests are performed under these unfavorable conditions in a cold room, indistinct, undependable results will be obtained.

## Notes on False Positive Reactions

In spite of the fact that the various accepted serologic tests for syphilis, such as the Kahn, Kolmer, Eagle, Mazzini, and Hinton, are remarkably sensitive and specific, it is well recognized that false positive reactions do occur. In general, these false positives fall into three categories, namely, (1) false positives of a technical nature resulting from test performance errors or clerical errors; (2) false positives that may be associated with diseases other than syphilis, such as malaria, leprosy, infectious mononucleosis, typhus, acute respiratory infections, and those that may be associated with immunization processes; and (3) false positives without apparent associated cause, occasionally found in persons who seemingly are in the best of health.

Questionable serologic findings should always be confirmed by recheck tests to insure that an accurate and dependable serologic study of the patient's serum has been made. Technical false positives may occur in any laboratory; the frequency of their occurrence usually is proportional to the care excercised in the performance of the tests and rigid adherence to the prescribed technique.

It has been observed that the percentage of false positives associated with disease is higher when blood is drawn in the febrile state. It is important that it be recognized that these false positives are often transitory in nature and that the serologic reactions usually become negative as the patient improves and becomes afebrile. Quantitative determinations showing a progressive decrease in titer without treatment are strongly suggestive of false positive reactions. Interval retesting with an interval of from 10 days to 3 weeks, is at the present time the most practical method of studying reactions thought to be due to diseases other than syphilis.

Note: Positive serologic reactions occurring in cases of yaws, pinta, and bejel should not be regarded as false positives since these reactions are due to the presence in the tissues of spirochetes belonging to the genus Treponema. The diagnosis of any one of the treponematoses should be made upon the basis of clinical and epidemiological findings with whatever assistance serologic studies may give.

Dr. Kahn has advised that any serum giving reactive or weakly reactive Kahn results should undergo a confirmatory test. This test is best carried out by placing the heated serum in the refrigerator for 24 hours; then reheating the serum for 10 minutes at 56°C. and repeating the standard Kahn test. Generally, syphilitic serum will give the same result as previously, while a non-syphilitic serum is likely to give a much weaker or a negative result.

## SECTION V

## COMPLEMENT FIXATION

### Principles Involved in Complement Fixation Tests

Various modifications of the Wassermann reaction have been published. All complement fixation tests depend upon the same principle (fixation of the complement.) The only difference is in the antigen; the antigen must be specific for the amboceptor-like substance present in the serum.

### Principle of All Complement Fixation Tests

(Normal rabbit serum contains a complement (heat labile) and amboceptor (heat stable).)

1. Normal rabbit serum plus sheep cells equals no hemolysis.
   (heated or unheated)

2. Sensitized rabbit serum plus sheep cells equals hemolysis.
   (amboceptor plus complement)
   (rabbits sensitized by repeated injections with sheep cells)

3. Heated sensitized rabbit serum plus sheep cells equals no hemolysis.
   (amboceptor, no complement)
   (rabbit serum heated at 56°C. for 30 minutes inactivates the complement.)

4. Heated, sensitized rabbit serum plus guinea pig serum plus sheep cells
   (amboceptor) (complement)
   equals hemolysis.
   (Guinea pig serum is a most satisfactory source of complement; however any other unheated serum could be used.)

5. Application of the above principles to the complement fixation procedure:

Since this antigen-antibody-complement reaction is visible, it is sometimes referred to as the "INDICATOR SYSTEM." When, as in the Kolmer test the combination of antigen with reagin and complement is not visible, the Indicator System is of use as outlined below.

a. Antigen plus normal serum plus guinea pig serum plus sensitized rabbit serum
   (heated) (unheated) (heated)
   plus sheep cells equals hemolysis.

Nonluetic serum does not contain any amboceptor-like substance that will combine with the antigen, therefore the complement is not used in the first part of the reaction and is free to be used in the second part of the reaction, where it attaches the specific amboceptor in the rabbit serum to the sheep cells and produces hemolysis of the cells.

b. Antigen plus luetic serum plus complement plus sensitized rabbit serum plus sheep cells equals no hemolysis.

Luetic serum contains an amboceptor-like substance (in the serum globulin fraction called reagin) which is specific for the antigen, therefore the amboceptor combines with the antigen, using all the available complement from the guinea pig serum. When the sensitized rabbit serum (the hemolysin) is added plus the sheep cells, there is no available complement, therefore the amboceptor cannot produce hemolysis of the sheep cells.

The complement is used by the luetic serum and fixed to the antigen, leaving no complement available for hemolysis of the sheep cells. This is fixation of the complement.

In the complement fixation test the serum to be tested is heated (inactivated) to destroy its complement. The complement in the rabbit serum (hemolysin) has been destroyed. Therefore the only source of complement is the guinea pig serum which must be carefully titrated so that a four plus luetic serum will utilize all of the complement. A weak luetic serum will use only part of the complement, leaving some available to produce partial hemolysis of the sheep cells.

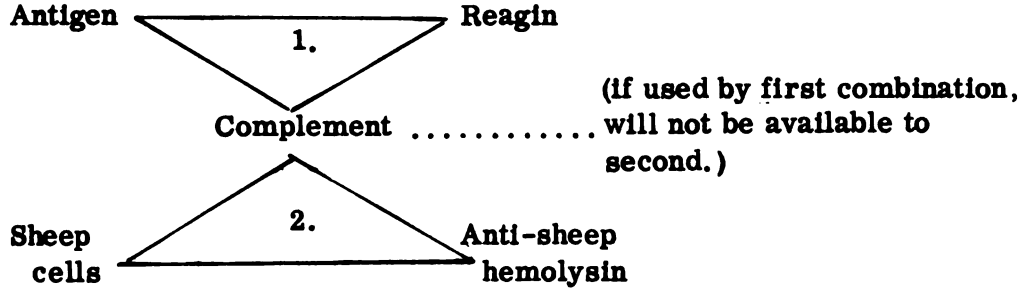



Fig. 1

Stock Reagents

Hemolysin: Anti-sheep hemolysin can be prepared by giving a rabbit five to six injections of 5 ml. of a 10 per cent suspension of washed sheep cells every three days. Bleed the rabbit 7 to 9 days after the last injection if a preliminary titration is satisfactory. Separate the serum (it need not be inactivated). Hemolysin is preserved by adding an equal volume of neutral glycerin to the rabbit serum. Employ the highest

grade of glycerin. This glycerinated stock can be stored for long periods of time in the refrigerator with little loss of activity.

Antigen There are two kinds of Kolmer Antigen. The lipoidal or older antigen is prepared from the alcohol soluble (acetone insoluble) fraction of beef heart. To this extract, cholesterol is added. The newer type of Kolmer Antigen is composed of cardiolipin, lecithin, and cholesterol dissolved in alcohol. Both antigens should be tightly stoppered and stored at room temperature in the dark. The titer of the antigen will be given on the label of the bottle.

Saline Solution

a. Weigh 8.5 gm. of dried sodium chloride (A.C.S.) and 0.1 gm. of magnesium sulfate for each liter of saline solution.[1]

b. Dissolve salts in distilled water, and filter into flasks having glass or gauze-covered cotton stoppers. Freshly prepared saline solution should be used for each test run.

c. Place portion of saline solution sufficient for diluting complement, to be used for completing the tests, into refrigerator, allowing remainder to stand at room temperature (73° to 85° F.).

Sheep Red Cells

1. The method of choice is to collect blood from an external jugular vein as asceptically as conditions permit. Otherwise fresh sheep blood should be collected at an abattoir.

2. Collection in an anticoagulant and preservative solution is recommended. For this purpose one part of the following solution (Boerner-Lukens) for each nine parts of blood is satisfactory:

| | |
|---|---|
| Sodium citrate ..... | 8.0 gm. |
| Dextrose ..... | 20.0 gm. |
| Aqueous solution of merthiolate, 1:1,000 .... | 100.0 ml. |

The following method (Kolmer) is likewise satisfactory: In a clean (but not necessarily sterile) quart-sized Mason jar, place 30 ml. of 10 per cent solution of sodium citrate in saline solution and 2 ml. of formalin. At the abattoir have the jar filled with fresh blood, screw on top, and mix thoroughly. A bottle containing A.C.D. solution, used for drawing blood from donors, is also satisfactory.

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[1]When unusually low complement titers are obtained, Dr. Kolmer recommends that 40 mg. of $CaCl_2 \cdot 2H_2O$ be added to each liter of saline solution. Under these circumstances, the complement should not be used at a dilution higher than 1:37 (for 2 full units).

Another satisfactory method is to dissolve 3.8 gm. of sodium citrate (A.C.S.) in 100 ml. of distilled water. Sixty ml. of this solution is required for each 50 ml. of sheep blood collected. If sterile technic is employed in collecting and dispensing the cells which are stored at refrigerator temperature, in sterile vaccine bottles equipped with sterile rubber stoppers, the cells may remain satisfactory for use over a 3 month period.

3. Sheep cells collected by any of these methods should be kept in the refrigerator for 48 hours before they are used in a test.

Complement is known more by what it does, than by what it actually is. Found in the blood of various species of animals, the complement in the serum of the guinea pig is most widely used. Of the four known components of complement, the best proportions of each are present in the guinea pig sera. Hemolysis requires the united effort of all four components.

Complement is a very labile substance. When submitted to a temperature of 56°C. a sudden drop in activity occurs, but a 20 to 30 minute period of heating at that temperature is employed for the patient's sera undergoing tests. As it is believed that sera on standing may regain some of their complement activity, they must be reinactivated for 5 minutes on the day they are tested. Agitation, chemicals (acids, alkalis), and dilution also decrease the potency of complement.

For routine use, the following procedures assist in overcoming the deleterious effects mentioned in the above paragraph, and assist in keeping the complement as active as possible. The stock vial of complement is kept in the refrigerator in a beaker of cold water. Whenever any is required for titration, the amount is removed and the vial immediately returned to the refrigerator. For actual tests, such as the Kolmer, the calculated dilution of complement in amount necessary for the tests being run is prepared with cold saline just prior to its addition to the serum. This precedes the overnight incubation at 4°C. in the Kolmer technic.

## Preparation and Preservation of Complement

Guinea pig blood may possess complement activity less than the prescribed minimum or greater than the prescribed maximum. Low complement titers may be caused by improper feeding or housing of guinea pigs or, most commonly, through loss of reactivity during storage of guinea pig serum. Complement serum stored as liquid (with preservative added) at refrigerator temperature or in the frozen state should be adequately protected from partial drying as a result of evaporation. Aliquots sufficient for 1 day's use should be placed in closed containers to avoid complement destruction due to repeated thawing and refreezing.

Some technicians are deceived by restoring dehydrated complement serum to only one-half or two-thirds of the original serum volume and then omitting the serum concentration factor when calculating complement dilution. Substandard serum may be made to appear adequately reactive in this way. This practice, used to circumvent technique restrictions, is to be discouraged.

### Preparation

1. As shown by Giordano and Carlson (Am. J. Clin. Path. 9: 130, 1939), it is advisable to pretest serums of guinea pigs individually for nonspecific reactions before use as complement in conducting complement fixation tests for syphilis. These preliminary tests should be conducted with the same lipoidal antigen as employed in the test for syphilis because, as shown by Harris (J. Lab. & Clin. Med. 27: 97, 1941), nonspecific reactions may occur with one antigen and not with another. Mixtures of serums of 100 or more guinea pigs, however, are rarely unsatisfactory. When such are used, the complement may be pretested after pooling. If found to yield nonspecific reactions with one antigen, it should be tested with others as it is extremely unlikely that any pools will be found that are not satisfactory with at least one of them. The technique employed for pretesting the complement for the Boerner-Lukens and Kolmer complement fixation tests is described below.

2. The pooled serums of at least three to five healthy young guinea pigs should be used. Select large, well-nourished animals that have not been fed for at least 12 hours; avoid pregnant animals.

3. An excellent and economical procedure is to maintain a colony of guinea pigs and to remove 4 ml. to 5 ml. of blood from the hearts of a sufficient number to yield the required amount of complement serum. The animals may be bled in rotation every 4 to 6 weeks. Otherwise the animal may be exsanguinated as follows: Anesthetize the guinea pig lightly with ether or stun the animal with one or two sharp blows on the head; sever the large blood vessels on both sides of the neck, be careful not to cut the esophagus or trachea; and collect the blood in centrifuge tubes by means of a large funnel. With either method Kolmer advises placing the blood in an

incubator at 37°C. for an hour before the clots are rimmed and centrifuged for collection of serum. Otherwise as an alternate method the clot may be kept at room temperature for one or two hours and then placed in the refrigerator overnight for serum separation the next morning.

4. Pretesting of complement includes the following technique for testing each guinea pig serum:

a. Prepare a 1:30 dilution by mixing 0.15 ml. with 4.35 ml. of saline solution.

b. In a series of six tubes place 0.8 ml., 0.6 ml., 0.4 ml., 0.8 ml., 0.6 ml., and 0.4 ml., respectively.

c. To each of the first three tubes add 0.5 ml. of the same antigen to be used in the complement titration and the complement fixation tests so diluted as to carry the optimum dose.

d. Add saline solution to a total volume of 2.0 ml. in each tube, as shown. Arrange six tubes for each complement in the following manner:

Table 1.

| | Tube 1 | Tube 2 | Tube 3 | Tube 4 | Tube 5 | Tube 6 |
|---|---|---|---|---|---|---|
| Complement, 1:30 | 0.8 ml. | 0.6 ml. | 0.4 ml. | 0.8 ml. | 0.6 ml. | 0.4 ml. |
| Kolmer antigen | 0.5 ml. | 0.5 ml. | 0.5 ml. | - | - | - |
| Saline | 0.7 ml. | 0.9 ml. | 1.1 ml. | 1.2 ml. | 1.4 ml. | 1.6 ml. |

e. Mix and place in refrigerator at 6° to 8°C. for 15 to 18 hours, or overnight, followed by 10 minutes in a water bath at 37°C.

f. Add 0.5 ml. of hemolysin (2 units), and 0.5 ml. of a 2 per cent suspension of sheep cells to each tube.

g. Mix and place in a water bath at 37°C. for 1 hour and examine.

h. Serums showing complete hemolysis in all six tubes are entirely satisfactory. Serums showing an equal degree of inhibition of hemolysis in the first three tubes having antigen as compared to the last three tubes having no antigen may be used but will usually be found to have a low hemolytic activity in the complement titration. Any serum showing a greater degree of inhibition of hemolysis in the first three tubes having the antigen than in the last three tubes not having antigen should be regarded as unsatisfactory for the complement titration and complement fixation tests insofar as the particular antigen employed is concerned. Such serums may be satisfactory with another Kolmer antigen.

Cardiolipin-lecithin antigens are free of the fraction responsible for the nonspecific fixation of complement at low temperature. Pretesting of guinea pig serums is therefore omitted when this type of antigen is employed.

## Preservation

Method 1. The most satisfactory method is to evaporate 5 ml. amounts in a vacuum by lypholization or a cryochemical technique. This serum usually retains both hemolytic activity and fixability for 8 to 12 months if kept under constant refrigeration. By adding 5 ml. of distilled water the material is ready for use in the same manner as fresh serum.

Method 2. Rhamy's sodium acetate method as modified by Sonnenschein (Ztschr. f. Immunitatsforsch. u. Exper. Therap. 67: 512, 1930) may be employed by adding to the complement serum an equal amount of solution prepared by dissolving 12 gm. of sodium acetate and 4 gm. of boric acid in 100 ml. of distilled water. Thus, 2 ml. diluted with 8 ml. of saline solution gives a 1 to 10 dilution of complement or 1 ml. diluted with 14 ml. of saline solution a 1:30 dilution. Store in refrigerator.

Method 3. Add 1.0 gm. of sodium chloride for every 10 ml. of guinea pig serum. Store in refrigerator.

Method 4. Freeze complement serum and retain in the frozen state until used.

## The Procedure for the Kolmer Test

### Equipment

1. Galvanized wire test tube racks (72 tube capacity).
2. Waterbath (56°C.)
3. Waterbath (37°C.)
4. Refrigerator (6.0°C.-10°C.)
5. Centrifuge

### Glassware

1. Test tubes, Pyrex, 15 mm. x 85 mm.
2. Test tubes, centrifuge, graduated, 15 ml. capacity, Pyrex.
3. Tubes, centrifuge, round bottom, 50 ml. capacity.
4. Pipets, serological, calibrated to tip.
   a. 1 ml. calibrated in 1/100 ml.
   b. 5 ml. calibrated in 1/10 ml.
   c. 10 ml. calibrated in 1/10 ml.

### Reagents

1. Antigen
   There are two types of antigen available at this time.
   a. The old lipoidal type of antigen.

b. The more recently developed antigen which is an alcoholic solution containing cardiolipin, lecithin, and cholesterol.

In each case, a new lot of antigen should be tested in parallel with a standard antigen, in both qualitative and quantitative tests with reactive, weakly reactive and non-reactive sera before being placed in routine use.

2. Saline Solution

Refer to Stock Reagents (page 34).

3. Sheep Cells

Refer to Stock Reagents (page 34).

4. Hemolysin

Refer to Stock Reagents (page 33).

5. Complement

a. Dehydrated complement should be restored to original serum volume by dissolving in the proper amount of buffered diluent or distilled water and then stored in the refrigerator. (If the commercial source added a preservative prior to lyophilization, no diluent will accompany the vial of dehydrated serum).

b. Complement stored in the frozen state should be returned to the liquid state by incubating at room temperature or at 37°C. only long enough to melt. Since the proteins in this serum will tend to precipitate during thawing the tubes should be adequately mixed by inversion prior to refrigeration.

Preparation of Serum

1. The specimens are properly labeled.

2. The specimens are centrifuged and the serums separated into properly labeled test tubes.

3. The serum is inactivated by placing the tubes in a water bath at 56°C. for 20 to 30 minutes. If serum has been heated a day or two before the actual test, it should be reheated for 5 minutes at 56°C. before proceeding with the test.

4. Recentrifuge any serum in which visible particles have formed during heating.

Note: If complement fixation reactions of maximum sensitivity are desired in the Kolmer quantitative test, it is necessary to remove the natural anti-sheep hemolysins from the serum. This may be accomplished in the following manner:

a. Pipet 1 ml. of each serum into a small (12 x 75 mm.) Kahn tube and place in the refrigerator for 15 minutes, or more.

b. Add one drop of washed, packed, sheep red cells to each specimen and mix well.

c. Return all tubes to the refrigerator for 15 minutes.

d. All tubes are then centrifuged and the serum separated by decanting. Avoid carrying over cell residue from side walls and bottom.

e. These sera are then heated at 56°C. for 30 minutes. Previously heated, absorbed sera should be reheated for 5 minutes.

## Preparation of Spinal Fluid

1. Centrifuge and decant all spinal fluids to remove cellular and particulate debris. Spinal fluids which are visibly contaminated or contain gross blood should not be tested.

2. Heat all spinal fluids received through the mail, or stored for three or more days, at 56°C. for 15 minutes to remove the thermolabile anticomplementary substances.

3. Fresh spinal fluids are tested without preliminary heating.

## Preparation of Sheep Red Cell Suspension

1. Filter an adequate quantity of preserved sheep blood through gauze into a 50 ml. round bottom centrifuge tube.

2. Add two or three volumes of saline solution to each tube. Invert to mix.

3. Centrifuge tubes at a force sufficient to pack the red cells in 5 minutes. (I.E.C.[2] centrifuge No. 1 at 2,000 rpm, I.E.C. centrifuge No. 2 at 1,700 rpm.)

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[2] International Equipment Co., Boston, Mass.

4. Remove supernatant fluid by suction through a capillary pipet, taking off also the upper white cell layer.

5. Fill tube with saline solution and resuspend cells by inverting and gently shaking tube. Invert until all cells are off the bottom.

6. Recentrifuge tube and repeat the process for a total of three washings. If supernatant fluid is not colorless on third washing, cells are too fragile and should not be used.

7. After supernatant fluid is removed from third washing, cells are poured or washed into a 15 ml. graduated centrifuge tube and centrifuged at previously used speed for 10 minutes in order to pack cells firmly and evenly.

8. Read the volume of packed cells in the centrifuge tube and carefully remove supernatant fluid. Try not to disturb cell layer.

9. Prepare a 2 per cent suspension of sheep cells by washing them into a flask with 49 volumes of saline solution. Shake flask to insure even suspension of cells.

Example: 2.1 ml. (packed cells) x 49 = 102.9 ml. (saline solution required).

10. Pipet 15 ml. of the 2 per cent cell suspension into a graduated centrifuge tube and centrifuge at previously used speed for 10 minutes. A 15 ml. aliquot of a properly prepared cell suspension will produce $0.3 \pm 0.01$ ml. of packed cells.

Caution: Use only centrifuge tubes which have been properly calibrated and checked for accuracy.

Note: When the packed cell volume is beyond the tolerable limits stated above, the cell suspension concentration should be adjusted. The quantity of saline solution which must be removed or added to the cell suspension to accomplish this is determined according to the following formula:

$$\frac{\text{Actual reading of centrifuge tube}}{\text{Correct reading of centrifuge tube}} \times \text{Volume of cell suspension} = \text{Corrected volume of cell suspension}$$

**Example 1:**

Volume of cell suspension ..... 100 ml.
Centrifuge tube (15 ml.) reading ..... 0.27 ml.

$$\frac{0.27 \text{ ml.}}{0.3 \text{ ml.}} \times 100 \text{ ml.} = 90 \text{ ml.}$$

Therefore, 10 ml. of saline solution should be removed from each 100 ml. of cell suspension. Saline solution may be removed by centrifuging an aliquot of the cell suspension and pipetting off the desired volume of saline solution for discard.

**Example 2:**

Volume of cell suspension ..... 100 ml.
Centrifuge tube (15 ml.) reading..... 0.33 ml.

$$\frac{0.33 \text{ ml.}}{0.3 \text{ ml.}} \times 100 \text{ ml.} = 110 \text{ ml.}$$

Therefore, 10 ml. of saline solution should be added to each 100 ml. of cell suspension. An adjusted cell suspension should be rechecked by centrifuging a 15 ml. portion.

11. Place flask of cell suspension in refrigerator when not in use. Always shake before using to secure an even suspension, since the red cells settle to the bottom of the flask when allowed to stand.

**Preparation of Antigen Dilution**

1. Place the required amount of saline solution in a flask and add antigen drop by drop while continuously shaking the flask. The amount needed may be calculated from the number of tubes containing antigen in the tests and titrations. The test dose constitutes 0.5 ml. of the antigen dilution indicated on the label of the bottle.

2. Diluted antigen is kept at room temperature in a stoppered flask.

3. Diluted lipoidal antigen should stand ten minutes before use, BUT diluted cardiolipin antigen should stand at room temperature for at least ONE HOUR before it is used. During this interval the cardiolipin antigen will become more opalescent. The titer of cardiolipin antigen is usually 1:150 (commercial sources) or 1:130 (if procured from the Army).

| Formula of: | Alcoholic solution | Cardiolipin | Lecithin | Cholesterol |
|---|---|---|---|---|
| Kolmer's cardiolipin antigen | yes | 0.03% | 0.05% | 0.30% |
| Army's cardiolipin antigen for C.F. | yes | 0.0175% | 0.0875% | 0.30% |

Note: The complement fixation tests for serum and cerebrospinal fluid using the Army's cardiolipin antigen are carried out exactly as directed by Kolmer except that the working antigen emulsion is prepared by measuring the required amount of Kolmer saline into a beaker of suitable size, and then measuring the required amount of antigen (use a pipet) into the bottom of a second identical beaker following which the saline solution is then added rapidly to the antigen, and the two mixed thoroughly by pouring 6 times from one beaker to the other. The diluted antigen is then ready for use. It may be kept at room temperature, or preferably in the refrigerator (3-6°C.) but should be used only on the day prepared. (Not less than 0.2 ml. of antigen should be used in preparing the dilution. Mixing 25.8 ml. of saline solution with 0.2 ml. of antigen gives 26 ml. of the optimal 1:130 dilution; larger amounts may be prepared by mixing proportionally greater volumes of antigen and saline solution.)

Preparation of Stock Hemolysin Dilution

1. Prepare 1:100 stock hemolysin dilution as follows:
   Saline solution ..... 94.0 ml.
   Phenol solution (5 per cent in saline solution) ..... 4.0 ml.
   Glycerinized hemolysin (50 per cent) ..... 2.0 ml.

Phenol solution should be mixed well with the saline solution before glycerinized hemolysin is added. This solution keeps well at refrigerator temperature but should be discarded when found to contain precipitate.

2. Each new lot of stock hemolysin dilution (1:100) should be checked by parallel titration with the previous stock hemolysin dilution before it is placed into routine use.

3. Dilutions of hemolysin of 1:1,000 or greater are prepared by further diluting aliquots of the 1:100 dilution.

After these reagents are prepared the complement and hemolysin titrations may be assembled.

Complement and Hemolysin Titrations

1. Perform these two titrations simultaneously in the same rack.

2. Place 10 tubes (numbered 1 to 10) in one side of the rack for the hemolysin titration and 8 tubes (numbered 1 to 8) in the other side for the complement titration. Add two other tubes to the rack, one for 1:1.000 hemolysin dilution and one for 1:30 complement dilution.

3. Prepare a 1:1,000 dilution of hemolysin by placing 4.5 ml. of saline solution in a test tube and adding 0.5 ml. of 1:100 stock hemolysin solution. Mix well.

4. Pipet 0.5 ml. of 1:1,000 hemolysin dilution into the first five tubes of the hemolysin titration.

5. Add the following amounts of saline solution to the hemolysin titration tubes:

Table 2.

| Tube Number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Saline solution | None | 0.5 ml. | 1.0 ml. | 1.5 ml. | 2.0 ml. | 0.5 ml. | 0.5 ml. | 0.5 ml. | 0.5 ml. | 0.5 ml. |

(For speed and accuracy in the addition of saline a 5.0 ml. (graduated serological) pipet should be used. The readings of the pipet upon successive deliveries of saline starting with tube 2 through 4 are: 0.5, 1.5, 3.0. Refill the pipet, add 2.0 ml. to tube 5 and 0.5 ml. to the rest of the tubes. In serological technic, it is not good practice to use the last 0.5 ml. of fluid at the tip of the pipet.)

6. Proceed as follows:

Table 3.

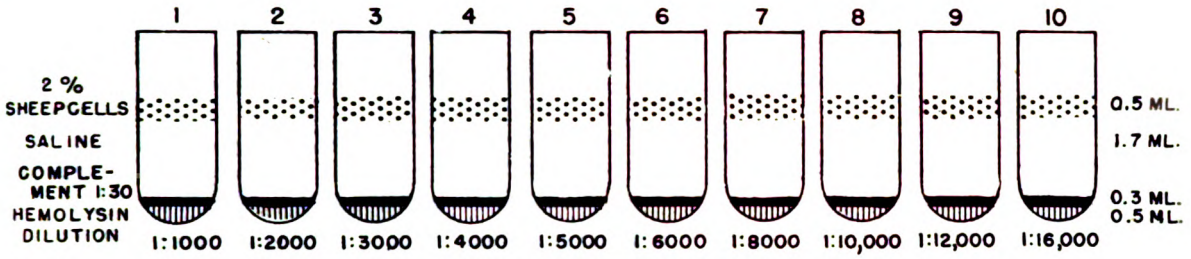
| Tube No. | Process | Final hemolysin dilution |
|---|---|---|
| 1 | Mix. None | 1:1,000 |
| 2 | Mix. Discard 0.5 ml. | 1:2,000 |
| 3 | Mix. Transfer 0.5 ml. to tube 6. Discard 0.5 ml. | 1:3,000 |
| 4 | Mix. Transfer 0.5 ml. to tube 7. Discard 1.0 ml. | 1:4,000 |
| 5 | Mix. Transfer 0.5 ml. to tube 8. Discard 1.5 ml. | 1:5,000 |
| 6 | Mix. Transfer 0.5 ml. to tube 9. | 1:6,000 |
| 7 | Mix. Transfer 0.5 ml. to tube 10. | 1:8,000 |
| 8 | Mix. Discard 0.5 ml. | 1:10,000 |
| 9 | Mix. Discard 0.5 ml. | 1:12,000 |
| 10 | Mix. Discard 0.5 ml. | 1:16,000 |

To assure a good mixing of the serum and saline, it is good technic to draw up the serum-saline mixture into the pipet and expel it back into the tube at least three times.

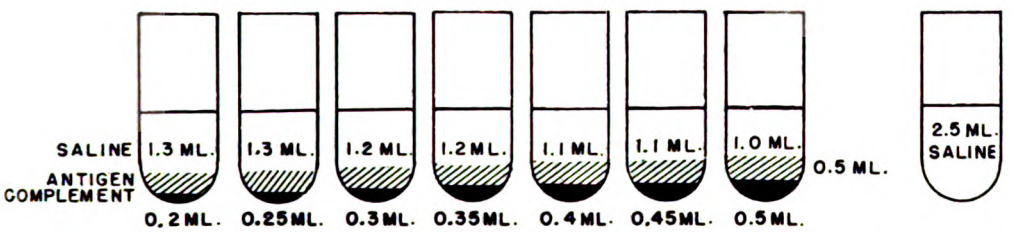
In making the transfers beginning with tube #3 to tube #6, tube #4 to #7 etc., each time two tubes are skipped. By placing a finger over the mouths of the tubes to be skipped, the chance of error due to incorrect dilution is reduced.

## SCHEMATIC REPRESENTATION

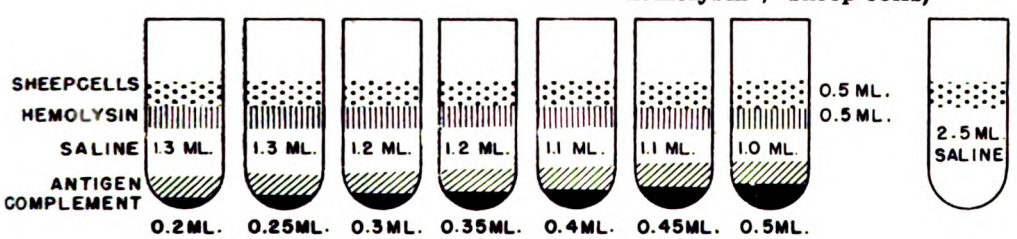
### I. HEMOLYSIN TITRATION




### II. A. COMPLEMENT TITRATION - First Stage

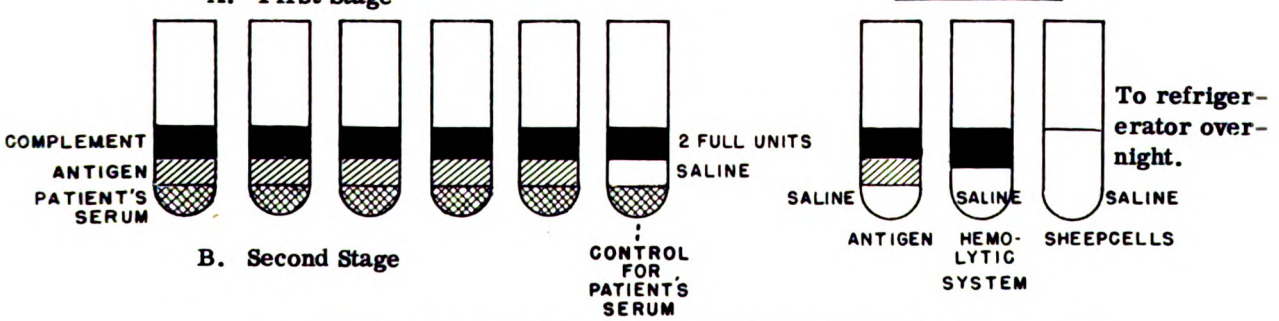



### B. COMPLEMENT TITRATION - Second Stage (incubation with hemolysin + sheep cells)



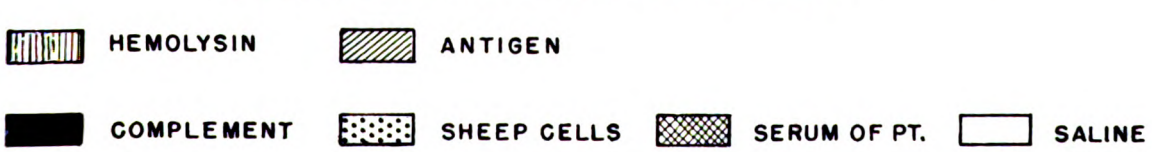

### III. KOLMER COMPLEMENT FIXATION TEST

#### A. First Stage




#### B. Second Stage

ADD HEMOLYSIN (2 UNITS) TO ALL TUBES EXCEPT SHEEP CELL CONTROL. ADD SHEEP CELLS TO ALL TUBES.



7. Prepare 1:30 dilution of complement by adding 0.2 ml. of guinea pig serum to 5.8 ml. of saline solution and mixing well.

Use a 5 ml. pipet. Draw saline to the 0.0 mark and release 4 ml. into tube. Refill pipet and add 1.8 ml. to the tube thereby avoiding using the last 0.5 ml. at the tip of the pipet.

8. Pipet 0.3 ml. of 1:30 complement into each of 10 tubes of the hemolysin titration.

9. Add the following amounts of 1:30 complement to the complement titration tubes.

Table 4.

| Tube Number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Complement 1:30 | 0.2 ml. | 0.25 ml. | 0.3 ml. | 0.35 ml. | 0.4 ml. | 0.45 ml. | 0.5 ml. | 0.0 ml. |

Successive readings of the 1.0 ml. (graduated serological) delivery pipet will be 0.2, 0.45, 0.75. Refill the pipet for delivery readings of 0.35, 0.75. Refill the pipet for delivery readings of 0.45 and 0.95.

10. Add 0.5 ml. of antigen dilution to each of the first seven tubes of the complement titration.

11. Add 1.7 ml. of saline solution to each of the 10 tubes of the hemolysin titration. For speed in the addition of 1.7 ml. of saline to each tube, a 10 ml. (graduated serological) pipet is used. This is the only time in the Kolmer test where the use of a 10 ml. pipet is advised. Readings upon delivery of 1.7 ml. of saline to successive tubes will be 1.7, 3.4, 5.1, 6.8, 8.5. Refill the pipet and repeat.

12. Add the following amounts of saline solution to the complement titration tubes.

Table 5.

| Tube Number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Saline Solution | 1.3 ml. | 1.3 ml. | 1.2 ml. | 1.2 ml. | 1.1 ml. | 1.1 ml. | 1.0 ml. | 2.5 ml. |

For addition of saline a 5.0 ml. (graduated serological) pipet is employed. The readings of the pipet on progressive delivery of saline to the respective tubes will be 1.3, 2.6, 3.8. Refill the pipet and deliver 1.2 to tube 4, next reading for tube 5 and successive deliveries will be 2.3, 3.4, 4.4. Refill the pipet to deliver 2.5 ml. to tube 8.

13. Add 0.5 ml. of 2 per cent sheep red cell suspension to each tube of the hemolysin titration.

14. Shake each tube of the hemolysin titration to insure even distribution of cells and place rack containing the two titrations in the 37°C. water bath for 1 hour.

At this point, the complement titration and the completed hemolysin titration stand as shown in Tables 6 and 7.

Table 6. Complement titration (first stage)

| Tube No. | Complement | Antigen | Saline |
|---|---|---|---|
| 1 | 0.2 | 0.5 | 1.3 |
| 2 | 0.25 | 0.5 | 1.3 |
| 3 | 0.3 | 0.5 | 1.2 |
| 4 | 0.35 | 0.5 | 1.2 |
| 5 | 0.4 | 0.5 | 1.1 |
| 6 | 0.45 | 0.5 | 1.1 |
| 7 | 0.5 | 0.5 | 1.0 |
| 8 | 0 | 0 | 2.5 |

Table 7. Hemolysin titration (complete)

| Tube No. | Hemolysin | Complement | Saline | Sheep Cell suspension -2% |
|---|---|---|---|---|
| 1 | 1:1,000 | 0.3 | 1.7 | 0.5 |
| 2 | 1:2,000 | 0.3 | 1.7 | 0.5 |
| 3 | 1:3,000 | 0.3 | 1.7 | 0.5 |
| 4 | 1:4,000 | 0.3 | 1.7 | 0.5 |
| 5 | 1:5,000 | 0.3 | 1.7 | 0.5 |
| 6 | 1:6,000 | 0.3 | 1.7 | 0.5 |
| 7 | 1:8,000 | 0.3 | 1.7 | 0.5 |
| 8 | 1:10,000 | 0.3 | 1.7 | 0.5 |
| 9 | 1:12,000 | 0.3 | 1.7 | 0.5 |
| 10 | 1:16,000 | 0.3 | 1.7 | 0.5 |

15. Remove rack from water bath and read hemolysin titration.

The unit of hemolysin is the highest dilution that gives complete sparkling hemolysis.

Hemolysin for the complement titration and test proper is diluted so that 2 units are contained in 0.5 ml.

16. Prepare a quantity of diluted hemolysin, containing 2 units per 0.5 ml. sufficient for the complement titration and the tests proper plus the controls in accordance with Table 8. Place the diluted hemolysin in the refrigerator when not in use.

Table 8. Preparation of Hemolysin Dilution

| Dilution containing 1 unit per 0.5 ml. | Dilution containing 2 units per 0.5 ml. | To prepare 2 unit hemolysin dilution | |
|---|---|---|---|
| | | 1:100 Hemolysin | Saline solution |
| 1:4,000 ..... | 1:2,000 | 0.3 | 5.7 |
| 1:5,000 ..... | 1:2,500 | 0.2 | 4.8 |
| 1:6,000 ..... | 1:3,000 | 0.2 | 5.8 |
| 1:8,000 ..... | 1:4,000 | 0.15 | 5.85 |
| 1:10,000 ..... | 1:5,000 | 0.1 | 4.9 |
| 1:12,000 ..... | 1:6,000 | 0.1 | 5.9 |
| 1:16,000 ..... | 1:8,000 | 0.1 | 7.9 |

17. Add 0.5 ml. of diluted hemolysin (containing 2 units of hemolysin) to each of the first seven tubes of the complement titration.

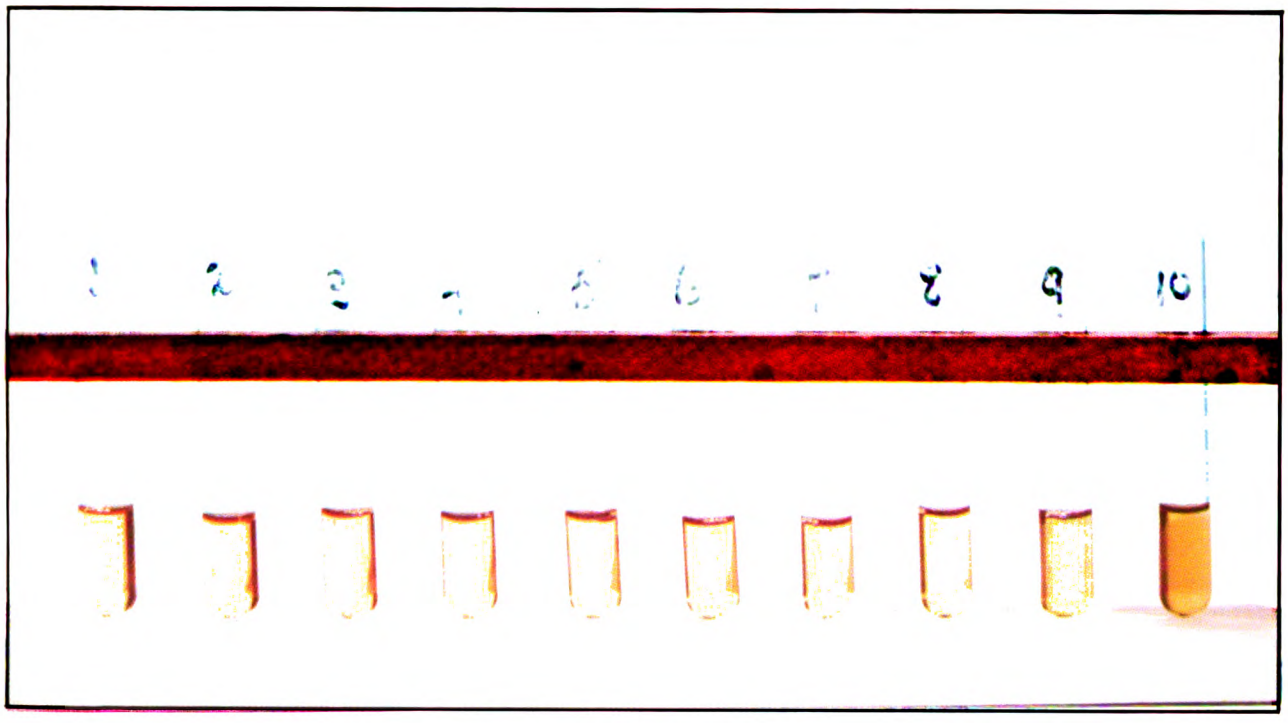
18. Add 0.5 ml. of 2 per cent sheep red cell suspension to all eight tubes of the complement titration. The addition of hemolysin and cells to the complement titration should be completed without delay, preferably within 5 minutes after rack is removed from the water bath.

19. Shake each tube of the complement titration to insure even distribution of cells and return to the 37°C. water bath for 1 hour.

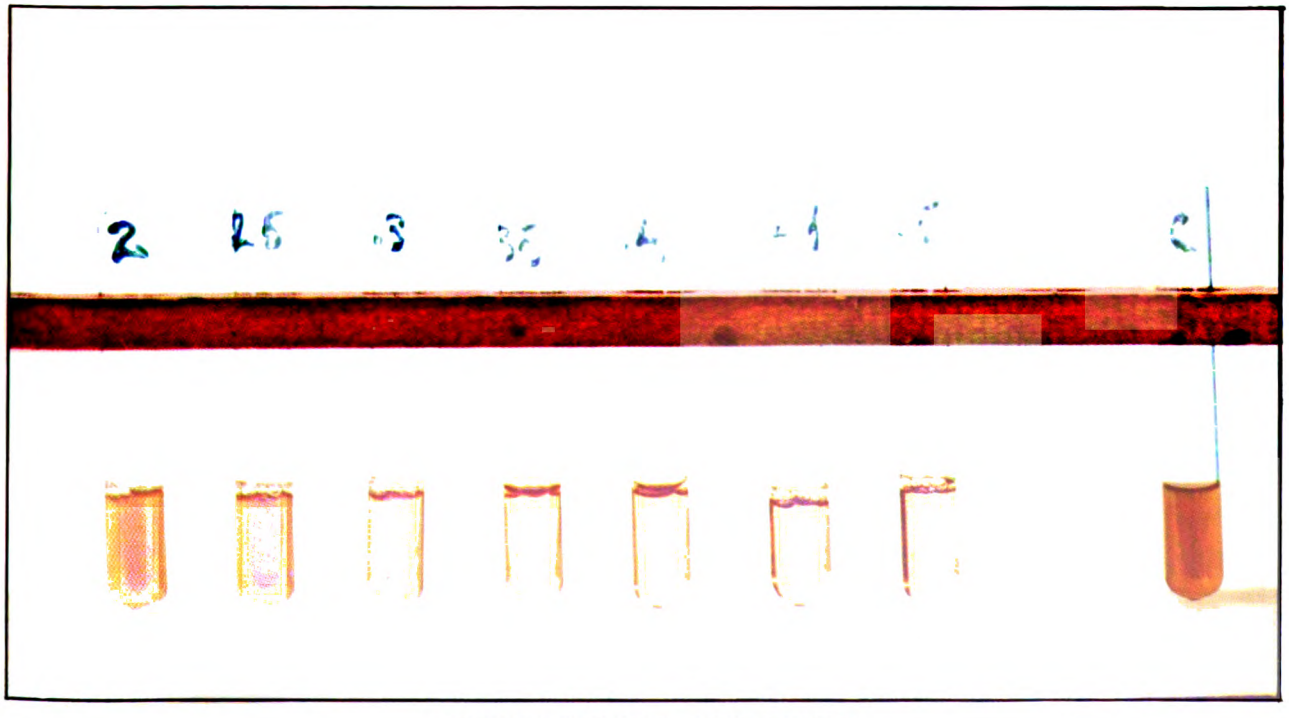
The completed complement titration is shown in Table 9.

Table 9. Complement titration (complete)

| Tube No. | Complement 1:30 | Antigen solution | Saline solution | | Hemolysin | Sheep cell suspension (2 per cent) | |
|---|---|---|---|---|---|---|---|
| 1 ..... | 0.20 | 0.5 | 1.3 | 37° C. Water bath for 1 hour. | 0.5 | 0.5 | 37° C. water bath for 1 hour. |
| 2 ..... | 0.25 | 0.5 | 1.3 | | 0.5 | 0.5 | |
| 3 ..... | 0.30 | 0.5 | 1.2 | | 0.5 | 0.5 | |
| 4 ..... | 0.35 | 0.5 | 1.2 | | 0.5 | 0.5 | |
| 5 ..... | 0.40 | 0.5 | 1.1 | | 0.5 | 0.5 | |
| 6 ..... | 0.45 | 0.5 | 1.1 | | 0.5 | 0.5 | |
| 7 ..... | 0.50 | 0.5 | 1.0 | | 0.5 | 0.5 | |
| 8 ..... | None | None | 2.5 | | None | 0.5 | |



HEMOLYSIN TITRATION



COMPLEMENT TITRATION

20. Remove rack from water bath and read complement titration.

The smallest amount of complement giving complete sparkling hemolysis is the exact unit. The full unit is 0.05 ml. more than the exact unit.

For the complement fixation tests, complement is diluted so that 2 full units are contained in 1.0 ml.

| Example: | ml. |
|---|---|
| Exact unit ..... | 0.3 |
| Full unit ..... | 0.35 |
| Dose (2 full units) ..... | 0.7 |

Dilution of complement to be employed in the test proper may be calculated by dividing 30 by the dose, i.e., $\frac{30}{0.7}$ = 43 or 1:43 dilution of complement (guinea pig serum).

Table 10 gives additional examples.

Table 10. Preparation of Complement Dilution

| Exact unit | Full unit | Dose | Dilution to use | Preparation | |
|---|---|---|---|---|---|
| | | | | Guinea pig serum | Saline solution |
| 0.3 | 0.35 | 0.7 | 1:43 | 1 | 42 |
| 0.35 | 0.4 | 0.8 | 1:37 | 1 | 36 |
| 0.4 | 0.45 | 0.9 | 1:33 | 1 | 32 |
| 0.45 | 0.50 | 1.0 | 1:30 | 1 | 29 |

Occasionally hyperactive guinea pig serums are encountered which yield titrations indicating 2 full units per milliliter in dilutions greater than 1:43. These guinea pig serums should be used at 1:43 dilution to accomplish satisfactory testing.

Note: Tubes of the complement or hemolysin titrations showing complete hemolysis may be removed and placed in the refrigerator for later use as hemoglobin solutions in preparing the reading standards.

Kolmer Qualitative Tests with Serum and Spinal Fluid

1. Arrange test tubes in wire racks so that there are two tubes for each serum and spinal fluid to be tested. Control serums of graded reactivity should be included. Number the first row of tubes to correspond to the serum and spinal fluid being tested. Four additional test tubes are included for reagent controls.

2. Pipet 0.5 ml. of saline solution into each tube of the second row.

3. Add the following amounts of saline solution to the four control tubes:

| | ml. |
|---|---|
| Antigen control (for serum tests) ..... | 0.2 |
| Antigen control (for spinal fluid tests) .... | 0.5 |
| Hemolytic system control ..... | 1.0 |
| Red cell control..... | 2.5 |

4. Pipet 0.2 ml. of each serum to be tested into tubes 1 and 2.

5. Pipet 0.5 ml. of each spinal fluid to be tested into tubes 1 and 2.

6. Pipet 0.2 ml. of each control serum to be tested into tubes 1 and 2.

7. Add 0.5 ml. of the antigen dilution to the first tube of each test, either serum, control serum or spinal fluid, and the two antigen control tubes.

8. Allow test racks to stand for 10 to 30 minutes at room temperature.

9. Prepare diluted complement during this interval. The amount needed is equivalent to 1.0 ml. for each tube of the test plus a slight excess.

Note: The volume of guinea pig serum to be diluted is determined by the amount of diluted complement necessary for the test proper. Dividing the number of milliliters of diluted complement needed by the titration dilution factor (2 full units) will give the number of milliliters of guinea pig serum needed. Calculations may be made in accordance with Table 11.

Table 11. Volume Determination Method

| Complement dilution required | Diluted complement required | Guinea pig serum required | Cold saline solution required |
|---|---|---|---|
| | ml. | ml. | ml. |
| 1:43 ..... | 43 | 1 | 42 |
| 1:43 ..... | 215 | 5 | 210 |
| 1:37 ..... | 37 | 1 | 36 |
| 1:30 ..... | 210 | 7 | 203 |

10. Add 1 ml. of diluted complement (containing 2 full units) to all tubes of the serum, control serum, and spinal fluid tests, including the antigen control tube and the hemolytic system control tube.

11. Mix the contents of the tubes by shaking the racks well and place in the refrigerator at 6°C. for 15 to 18 hours.

12. Remove racks of tubes from the refrigerator at regular intervals and place immediately in the 37°C. water bath for 10 minutes. The interval will be determined by the length of time necessary to add hemolysin and sheep cell suspension to each rack.

13. Remove each rack from the water bath and add 0.5 ml. of the diluted hemolysin to all tubes of the test except the red cell control tube.

14. Add 0.5 ml. of the 2 per cent sheep red cell suspension (prepared the previous day) to all tubes. The 2 per cent cell suspension should be agitated occasionally to insure even suspension of cells during the period when this reagent is being added to the complement fixation tests.

15. Mix the contents of the tubes thoroughly by shaking each rack before returning it to the 37°C. water bath for the secondary incubation. Examine the controls at 5 minute intervals. The period of secondary incubation will be determined by the length of time necessary to reproduce the predetermined reactivity pattern of the control serums. In all instances, however, the reading time should be at least 10 minutes more than is required to hemolyze the antigen and hemolytic system controls but should not exceed a total of 60 minutes incubation. When control serums are not available, the secondary incubation period will be terminated 10 minutes after the hemolytic system control and the antigen control hemolyze. In no instance should this period extend beyond 1 hour.

16. Remove each rack of tubes from the water bath at the end of the secondary incubation period. Record observed hemolysis as described under "Preparation of Reading Standards" and "Reading and Reporting Test Results" except in those instances where inhibition of hemolysis is noted in the control tube.

All serums and spinal fluids showing inhibition of hemolysis in the control tube should be returned to the 37°C. water bath for a period sufficient to complete 1 hour of secondary incubation. At the end of this period, these tests are removed from the water bath, and the tube readings (including control tubes) are recorded.

Table 12. Outline of Kolmer Qualitative Tests with Serum and Spinal Fluid

| Tube No. | Serum | Saline solution | Antigen | | Complement, 2 full units | | Hemolysin, 2 units | Sheep cell suspension (2 per cent) | |
|---|---|---|---|---|---|---|---|---|---|
| | ml. | ml. | ml. | | ml. | | ml. | ml. | |
| 1 | 0.2 | None | 0.5 | Shake rack well. Allow to stand at room temperature for 10 to 30 minutes. | 1.0 | Shake rack well. Primary incubation 15 to 18 hours at 6° to 8°C. followed by 10 minutes in 37°C. water bath. | 0.5 | 0.5 | Shake rack well. Secondary incubation in 37°C. water bath. |
| 2 | 0.2 | 0.5 | None | | 1.0 | | 0.5 | 0.5 | |
| | Spinal Fluid | | | | | | | | |
| 1 | 0.5 | None | 0.5 | | 1.0 | | 0.5 | 0.5 | |
| 2 | 0.5 | 0.5 | None | | 1.0 | | 0.5 | 0.5 | |
| Controls | | | | | | | | | |
| Antigen (serum) | | 0.2 | 0.5 | | 1.0 | | 0.5 | 0.5 | |
| Antigen (spinal fluid) | | 0.5 | 0.5 | | 1.0 | | 0.5 | 0.5 | |
| Hemolytic system | | 1.0 | None | | 1.0 | | 0.5 | 0.5 | |
| Red cell | | 2.5 | None | | None | | None | 0.5 | |

## Kolmer Quantitative Tests with Serum and Spinal Fluid using Cardiolipin Antigen

1. Place test tubes in wire racks, allowing 8 tubes for each serum and 6 tubes for each spinal fluid to be tested. Include reagent controls and control serums of graded reactivity.

2. For each serum, pipet 0.9 ml. of saline solution into tube 1 and 0.5 ml. of saline solution into tubes 2, 3, 4, 5, 6, 7, and 8.

3. For each spinal fluid, pipet 0.5 ml. of saline solution into tubes 2, 3, 4, 5 and 6.

4. Pipet the indicated amount of saline solution into each of the following reagent control tubes:

Antigen control..... 0.5 ml.
Hemolytic control ..... 1.0 ml.
Red cell control..... 2.5 ml.

5. For each serum proceed as follows:

Table 13.

| Tube No. | Process | Serum dilution |
|---|---|---|
| 1..... | Add 0.6 ml. of inactivated serum. Mix and transfer 0.5 ml. to tube 8 (control) and to tube 2. | Undiluted. |
| 2..... | Mix. Transfer 0.5 ml. to tube 3. | 1:2 |
| 3..... | Mix. Transfer 0.5 ml. to tube 4. | 1:4 |
| 4..... | Mix. Transfer 0.5 ml. to tube 5. | 1:8 |
| 5..... | Mix. Transfer 0.5 ml. to tube 6. | 1:16 |
| 6..... | Mix. Transfer 0.5 ml. to tube 7. | 1:32 |
| 7..... | Mix. Discard 0.5 ml. | 1:64 |
| 8..... | ..... | Undiluted (control) |

6. For each spinal fluid proceed as follows:

Table 14.

| Tube No. | Process | Spinal fluid dilution |
|---|---|---|
| 1..... | Add 0.5 ml. of spinal fluid. | Undiluted. |
| 2..... | Add 0.5 ml. of spinal fluid. Mix. Transfer 0.5 ml. to tube 3. | 1:2 |
| 3..... | Mix. Transfer 0.5 ml. to tube 4. | 1:4 |
| 4..... | Mix. Transfer 0.5 ml. to tube 5. | 1:8 |
| 5..... | Mix. Discard 0.5 ml. | 1:16 |
| 6..... | Add 0.5 ml. of spinal fluid. | Undiluted (control) |

7. Add 0.5 ml. of diluted antigen to the first seven tubes of each serum test, to the first five tubes of each spinal fluid test, and to the antigen control tube. Shake the racks to mix thoroughly.

8. Allow racks to stand at room temperature for 10 to 30 minutes.

9. Complete the tests as indicated in paragraphs 9 through 16 of the technique for the performance of the "Kolmer Qualitative Tests with Serum and Spinal Fluid."

Both qualitative and quantitative tests may be conducted in one-half volume. The amounts of complement, antigen, and other reagents needed are in this way reduced by half. Some accuracy of operation may be sacrificed by using these reduced quantities, however, since the relative effects of measuring errors are increased.

Hemolysin and complement are titrated at full volume as described for the regular test. The performance of the one-half volume tests is identical with the regular methods except that halved volumes of serum, spinal fluid, and reagents are used.

Table 15. Outline of Kolmer Quantitative Tests with Serum and Spinal Fluid

| Tube No. | | Antigen | | Complement 2 full units | | Hemolysin, 2 units | Sheep cell suspension (2 per cent) | |
|---|---|---|---|---|---|---|---|---|
| | Serum (in 0.5 ml.) | ml. | | ml. | | ml. | ml. | |
| 1 | 0.2 (undiluted) | 0.5 | | 1.0 | | 0.5 | 0.5 | |
| 2 | 0.1 (1:2) | 0.5 | | 1.0 | | 0.5 | 0.5 | |
| 3 | 0.05 (1:4) | 0.5 | | 1.0 | | 0.5 | 0.5 | |
| 4 | 0.025 (1:8) | 0.5 | | 1.0 | | 0.5 | 0.5 | |
| 5 | 0.012 (1:16) | 0.5 | | 1.0 | | 0.5 | 0.5 | |
| 6 | 0.006 (1:32) | 0.5 | | 1.0 | | 0.5 | 0.5 | |
| 7 | 0.003 (1:64) | 0.5 | | 1.0 | | 0.5 | 0.5 | |
| 8 | 0.2 (undiluted, control). | None | | 1.0 | | 0.5 | 0.5 | |
| | Spinal fluid (in 0.5 ml.) | | | | | | | |
| 1 | 0.5 (undiluted) | 0.5 | | 1.0 | | 0.5 | 0.5 | |
| 2 | 0.25 (1:2) | 0.5 | | 1.0 | | 0.5 | 0.5 | |
| 3 | 0.125 (1:4) | 0.5 | | 1.0 | | 0.5 | 0.5 | |
| 4 | 0.062 (1:82) | 0.5 | | 1.0 | | 0.5 | 0.5 | |
| 5 | 0.031 (1:16) | 0.5 | | 1.0 | | 0.5 | 0.5 | |
| 6 | 0.5 (undiluted, control) | None | | 1.0 | | 0.5 | 0.5 | |
| | Reagent controls | | | | | | | |
| Antigen, 0.5 ml. saline solution | | 0.5 | Shake rack well. Allow to stand at room temperature 10 to 30 minutes. | 1.0 | Shake rack well. Primary incubation 15 to 18 hours at 6° to 8°C. followed by 10 minutes in 37°C. water bath. | 0.5 | 0.5 | Shake rack well. Secondary incubation in 37°C. water bath. |
| Hemolytic, 1.0 ml. saline solution | | None | | 1.0 | | 0.5 | 0.5 | |
| Red cell, 2.5 ml. saline solution | | None | | None | | None | 0.5 | |

## Kolmer Quantitative Tests with Serum and Spinal Fluid Employing Lipoidal Antigen

The titration of complement and hemolysin follows the same procedure as outlined previously. The dilutions of spinal fluid are the same as those outlined with cardiolipin antigen. With lipoidal antigen five dilutions of serum plus a control tube are employed in the quantitative procedure, instead of the seven tubes plus a control tube outlined for the cardiolipin Kolmer procedure. With this lipoidal type of antigen, the need for egg albumin must be considered.

As shown by Boerner and Lukens (Am. J. Clin. Path. 11 (Tech. Suppl. 5: 71) 1941), the addition of 0.2 ml. of a 50 per cent solution of egg albumin in sterile saline solution to each tube of the regular quantitative and simplified tests employing spinal fluid is advisable for the prevention of prezone or nonspecific reactions. Kolmer and Lynch (Am. J. Clin. Path. 11: 402, 1941) have confirmed these observations and advise the addition of egg albumin routinely in conducting quantitative and simplified tests with spinal fluids although it may be omitted if the pretested complement is being employed and if prezone reactions are not being observed. They also advise its use in conducting quantitative serum tests, if and when, prezone reactions with normal serums (like - - 1 2 2) are being observed.

The egg albumin solution may be prepared as follows:

1. Break a fresh egg and separate the white from the yolk.

2. Pick out the heavy particles or filter through several layers of gauze.

3. Measure, beat briskly, and add an equal volume of sterile saline solution.

4. Keep in a refrigerator (may be used a week or two without the addition of a preservative).

An alternate method is to prepare a 10 per cent solution in sterile saline and to use this for diluting the complement so that 1 ml. carries two full units. This 10 per cent solution may be prepared by diluting 10 ml. of albumin with 90 ml. of sterile saline or by diluting 20 ml. of the 50 per cent solution with 90 ml. of sterile saline.

## Choice of Methods

The regular quantitative method employs five aliquots of serum or spinal fluid and is preferred when conditions permit, especially in testing the serums and spinal fluids of cases of syphilis in order to determine the response to treatment.

A three-tube qualitative test may be conducted with two aliquots of serum (0.2 ml. and 0.1 ml.). For economy, both tests may be conducted by using reagents in one-half amounts. This applies not only to the amounts of serum or spinal fluid but to the amounts of complement, antigen, hemolysin, and sheep cells employed.

Quantitative Complement Fixation Test

1. For each serum arrange six test tubes and place in them the following amounts of saline solution, respectively: 0.9 ml., 0.5 ml., 0.5 ml., 0.5 ml., 2.0 ml., and 0.5 ml. To the first tube add 0.6 ml. of inactivated serum. Mix and transfer 0.5 ml. to No. 2 and 0.5 ml. to No. 6 (control tube). Mix No. 2 and transfer 0.5 ml. to No. 3. Mix No. 3 and transfer 0.5 ml. to No. 4. Mix No. 4, transfer 0.5 ml. to No. 5, and discard 2.0 ml. after mixing.

2. For each spinal fluid arrange six test tubes and place 0.5 ml. of saline solution in tubes 2, 3, 4, 5, and 6. Add 0.5 ml. of spinal fluid to tubes 1, 2, and 6. Mix No. 2 and transer 0.5 ml. to No. 3. Mix No. 3 and transfer 0.5 ml. to No. 4. Mix No. 4 and transfer 0.5 ml. to No. 5. Mix No. 5 and discard 0.5 ml.

Add 0.2 ml. of egg albumin (50 per cent) to each of the six tubes and to the antigen control. (This may usually be omitted if the pretested complement is used.)

3. To the first five tubes of each test (serum or spinal fluid) add 0.5 ml. of diluted antigen having the proper dose and mix thoroughly.

4. Allow to stand for 10 to 30 minutes at room temperature.

5. Add 1 cc. of complement (two full units) to all tubes.

6. Include the following controls:

a. Antigen control containing 0.5 ml. of diluted antigen, 0.5 ml. of saline solution, 1 ml. of complement (two full units), and 0.2 ml. of egg albumin (50 per cent) if it has been used in the tests.

b. Hemolytic system control containing 1 ml. of saline solution and 1 ml. of diluted complement (two full units).

c. Red cell control containing 2.5 ml. of saline solution.

d. Controls of positive and negative serums are advisable.

7. Mix the contents of each tube by gentle shaking and place in a refrigerator at $6^{\circ}$ to $8^{\circ}$C. for 15 to 18 hours.

8. Place tubes in a water bath at 37°C. for 10 minutes (not longer).

9. To all tubes, except the red cell control, add 0.5 ml. of hemolysin (carrying two units) and 0.5 ml. of 2 per cent red cell suspension which has been well mixed.

10. Mix the contents of each tube by gentle but thorough shaking and place in a water bath at 37°C. Watch the serum, antigen, and hemolytic system controls and 10 minutes after these show complete hemolysis (usually 25 to 30 minutes) remove the tests and make the readings.

11. The following table shows the setup for the quantitative complement fixation test with serum when lipoidal (crude) antigen is employed:

Table 16. Quantitative test with serum

| Tube No. | Serum (in 0.5 ml.) | Antigen (ml.) | | Complement two full units (ml.) | | Hemolysin two units (ml.) | Red cell suspension 2 per cent (ml.) |
|---|---|---|---|---|---|---|---|
| 1.... | 0.2 ml. | 0.5 | Shake rack; room temperature 10 to 30 minutes. | 1.0 | Shake rack; refrigerate 15 to 18 hours; then place in 37°C. water bath for 10 minutes. | 0.5 | 0.5 |
| 2.... | 0.1 ml. | 0.5 | | 1.0 | | 0.5 | 0.5 |
| 3.... | 0.05 ml. | 0.5 | | 1.0 | | 0.5 | 0.5 |
| 4.... | 0.025 ml. | 0.5 | | 1.0 | | 0.5 | 0.5 |
| 5.... | 0.005 ml. | 0.5 | | 1.0 | | 0.5 | 0.5 |
| 6.... | 0.2 ml. (control) | None | | 1.0 | | 0.5 | 0.5 |
| | Controls | | | | | | |
| Antigen: | 0.5 ml. saline | 0.5 | | 1.0 | | 0.5 | 0.5 |
| Hemolytic: | 1.0 ml. saline | None | | 1.0 | | 0.5 | 0.5 |
| Red cell: | 2.5 ml. saline | None | | None | | None | 0.5 |

12. The following table shows the setup for the quantitative complement fixation test with spinal fluid when lipoidal antigen is employed:

Table 17. Quantitative test with spinal fluid

| Tube No. | Spinal fluid (in 0.5 ml.) | Egg albumin* 50 per cent (ml.) | Antigen (ml.) | Shake rack; room temp. 10 to 30 min. | Complement two full units (ml.) | Shake rack; refrig. 15 to 18 hrs. Then place in 37°C. water bath for 10 min. | Hemolysin two units (ml.) | Red cell suspension 2 per cent (ml.) |
|---|---|---|---|---|---|---|---|---|
| 1 ... | 0.5 ml. | 0.2 | 0.5 | | 1.0 | | 0.5 | 0.5 |
| 2 ... | 0.25 ml. | 0.2 | 0.5 | | 1.0 | | 0.5 | 0.5 |
| 3 ... | 0.125 ml. | 0.2 | 0.5 | | 1.0 | | 0.5 | 0.5 |
| 4 ... | 0.0625 ml. | 0.2 | 0.5 | | 1.0 | | 0.5 | 0.5 |
| 5 ... | 0.03125 ml. | 0.2 | 0.5 | | 1.0 | | 0.5 | 0.5 |
| 6 ... | 0.5 ml. (control) | 0.2 | None | | 1.0 | | 0.5 | 0.5 |
| | Controls | | | | | | | |
| Antigen: | 0.5 ml. saline | 0.2 | 0.5 | | 1.0 | | 0.5 | 0.5 |
| Hemolytic: | 1.0 ml. saline | None | None | | 1.0 | | 0.5 | 0.5 |
| Red cell: | 2.5 ml. saline | None | None | | None | | None | 0.5 |

*Can usually be omitted if pretested complement is employed.

Three-Tube Qualitative Test

Arrange three test tubes and place 0.9 ml., 0.5 ml., and 0.5 ml. saline solution in each, respectively. Add 0.6 ml. of serum to No. 1, mix, and transfer 0.5 ml. to tubes No. 2 and No. 3 (control). Mix tube No. 2 and discard 0.5 ml. Each tube now carries 0.5 ml. containing 0.2 ml., 0.1 ml., and 0.2 ml. serum, respectively. Antigen (0.5 ml.) is added to tubes No. 1 and No. 2 and the test completed in the same manner as the six-tube quantitative test. The same controls are used as in the quantitative test.

Methods Using One-Half Volumes

The hemolysin and complement are titrated and diluted in the same manner as in the regular methods. The amount of the former (carrying two units) is 0.25 ml. instead of 0.5 ml. and the amount of the latter (carrying two full units) is 0.5 instead of 1.0 ml. The dose of 2 per cent red cell suspension is 0.25 ml. instead of 0.5.

1. For each quantitative serum test arrange six tubes and transfer the following amounts of saline solution respectively: 0.45 ml., 0.25 ml., 0.25 ml., 0.25 ml., 1.0 ml., and 0.25 ml. Add 0.3 ml. of serum to tube 1, mix and transfer 0.25 ml. to tubes No. 2 and No. 6. Mix No. 2 and transfer 0.25 ml. to No. 3 and so on to No. 5 from which 1.0 ml. is discarded after mixing. Complete the test including antigen, hemolytic system, and red cell controls as shown in the next table. Control sera (known reactive and non-reactive) should be included.

Table 18. Modified quantitative test with serum

| Tube No. | Serum (in 0.25 ml.) | Antigen (ml. | | Complement two full units (ml.) | | Hemolysin two units (ml.) | Red cell suspension 2 per cent (ml.) |
|---|---|---|---|---|---|---|---|
| 1 ... | 0.1 ml. | 0.25 | Shake rack; room temperature 10 to 30 min. | 0.5 | Shake rack; refrigerate 15 to 18 hours; then place in 37°C. water bath for 10 minutes. | 0.25 | 0.25 |
| 2 ... | 0.05 ml. | 0.25 | | 0.5 | | 0.25 | 0.25 |
| 3 ... | 0.025 ml. | 0.25 | | 0.5 | | 0.25 | 0.25 |
| 4 ... | 0.0125 ml. | 0.25 | | 0.5 | | 0.25 | 0.25 |
| 5 ... | 0.0025 ml. | 0.25 | | 0.5 | | 0.25 | 0.25 |
| 6 ... | 0.1 ml. (control) | None | | 0.5 | | 0.25 | 0.25 |
| | Controls | | | | | | |
| Antigen: | 0.25 ml. saline | 0.25 | | 0.5 | | 0.25 | 0.25 |
| Hemolytic: | 0.5 ml. saline | None | | 0.5 | | 0.25 | 0.25 |
| Red cell: | 1.25 ml. saline | None | | None | | None | 0.25 |

2. For the three-tube qualitative test arrange three tubes and place 0.45 ml. of saline in tube No. 1 and 0.25 ml. in tubes No. 2 and No. 3. Add 0.3 ml. of inactivated serum to No. 1. Mix and transfer 0.25 ml. to No. 2 and to No. 3. Mix No. 2 and discard 0.25 ml. Antigen (0.25 ml.) is added to tubes No. 1 and No. 2 and the test completed in the same manner as the six-tube quantitative test. The same controls are used.

3. For each quantitative spinal fluid test arrange six test tubes and place 0.25 ml. of saline solution in tubes No. 2, 3, 4, 5 and 6. Add 0.25 ml. of spinal fluid to tubes No. 1, 2, and 6. Mix No. 2 and transfer 0.25 ml. to No. 3 and so on to No. 5, from which 0.25 ml. is discarded after mixing. Add 0.1 ml. of 50 per cent solution of egg albumin to all tubes, including the antigen control. Complete the test with antigen, hemolytic system, and red cell controls as shown in table above. It is advisable to include tests with known positive and negative spinal fluids as controls.

Table 19. Modified Quantitative Test with Spinal Fluid

| Tube No. | Spinal fluid (in 0.25 ml.) | Egg albumin* 50 per cent (ml.) | Antigen (ml.) | | Complement two full units (ml.) | | Hemolysin two units (ml.) | Red cell suspension 2 per cent (ml.) |
|---|---|---|---|---|---|---|---|---|
| | | | | Shake rack; room temperature, 10 to 30 min. | | Shake rack; refrigerate 15 to 18 hrs. Then 10 min. at 37°C. | | |
| 1 | 0.25 ml. | 0.1 | 0.25 | | 0.5 | | 0.25 | 0.25 |
| 2 | 0.125 ml. | 0.1 | 0.25 | | 0.5 | | 0.25 | 0.25 |
| 3 | 0.0625 ml. | 0.1 | 0.25 | | 0.5 | | 0.25 | 0.25 |
| 4 | 0.03125 ml. | 0.1 | 0.25 | | 0.5 | | 0.25 | 0.25 |
| 5 | 0.015625 ml. | 0.1 | 0.25 | | 0.5 | | 0.25 | 0.25 |
| 6 | 0.25 ml. (control) | 0.1 | none | | 0.5 | | 0.25 | 0.25 |
| | Controls | | | | | | | |
| Antigen: | 0.25 ml. saline | 0.1 | 0.25 | | 0.5 | | 0.25 | 0.25 |
| Hemolytic: | 0.5 ml. saline | none | none | | 0.5 | | 0.25 | 0.25 |
| Red cell: | 1.25 ml. saline | none | none | | none | | none | 0.25 |

*Can usually be omitted if pretested complement is employed.

Preparation of Reading Standards

1. Heat tubes of hemoglobin solution (saved from the titration or obtained from control tubes of current day's tests) in the 56°C. water bath for 5 minutes.

2. Prepare a 1:6 dilution of a 2 per cent red cell suspension by adding 5 ml. of saline solution to 1 ml. of 2 per cent suspension.

3. Prepare reading standards by mixing hemoglobin solution and cell suspension in the proportions given in table below.

Table 20. Preparation of Reading Standards

| Red Cell Suspension 1:6 | Hemoglobin solution | Equivalent complement fixation | |
|---|---|---|---|
| | | Per cent | Reading |
| (ml.) | (ml.) | | |
| 3.0 | 0.0 | 100 | 4+ |
| 1.5 | 1.5 | 50 | 3+ |
| 0.75 | 2.25 | 25 | 2+ |
| 0.3 | 2.7 | 10 | 1+ |
| 0.15 | 2.85 | 5 | ± |
| ... | 3.0 | 0 | - |

4. Reading standards are prepared with one-half volumes of cell suspension and hemoglobin solution when performing the one-half volume tests.

## Reading and Reporting Test Results

1. All serum and spinal fluid controls should show complete hemolysis.

2. Estimate the individual tube readings by comparison with the reading standards at the end of the secondary incubation period, and record degree of complement fixation noted, except for those specimens showing inhibition of hemolysis in the control tube.

3. Read the tubes that have been returned to the 37°C. water bath for a full hour's secondary incubation, estimating and recording the degree of complement fixation of each tube and control tube by comparison with the reading standards.

4. Report the results of the qualitative tests in accordance with the table below.

Table 21. Reporting Test Results

| Test tube reading | Control tube reading | Report | Test tube reading | Control tube reading | Report |
|---|---|---|---|---|---|
| 4+ | - | Reactive (positive) | 3+ | 3+ | Anticomplementary (A) |
| 3+ | - | Reactive (positive) | 3+ | 2+ | Anticomplementary (A) |
| 2+ | - | Reactive (positive) | 3+ | 1+ | Weakly Reactive (D) |
| 1+ | - | Reactive (positive) | 3+ | ± | Reactive (positive) |
| ± | - | Weakly reactive (D) | 2+ | 2+ | Nonreactive (neg.) |
| - | - | Nonreactive (neg.) | 2+ | 1+ | Nonreactive (neg.) |
| 4+ | 4+ | Anticomplementary (A) | 2+ | ± | Weakly Reactive (D) |
| 4+ | 3+ | Anticomplementary (A) | 1+ | 1+ | Nonreactive (neg.) |
| 4+ | 2+ | Weakly Reactive (D) | ± | ± | Nonreactive (neg.) |
| 4+ | 1+ | Reactive (positive) | | | |

D - doubtful; A - anticomplementary; neg. - negative

Note: The U.S. Public Health Service and many civilian laboratories are using the terminology, Reactive, Weakly Reactive, and Nonreactive, which is equivalent to the Navy terminology in the above table. Anticomplementary remains the same in both systems of reporting.

5. Quantitative tests are reported in terms of the highest dilution giving a 1+, 2+, 3+ or 4+ reading. The only time that a plus-minus (±) is considered in the titer is when it appears in the first tube of the series. In this case when all higher dilutions are negative the result is reported as weakly reactive (D).

There are two methods of reporting the results of the quantitative Kolmer test: Kolmer Units and "dils." If Kolmer Units are being used, then the results are reported as follows: (see page 62).

| | | | |
|---|---|---|---|
| First tube only (1+) | Positive, | one Kolmer Unit | (reactive) |
| First tube only (2+) | " | two Units | " |
| First tube only (3+) | " | three " | " |
| First tube only (4+) | " | four " | " |
| First two tubes | " | eight " | " |
| First three tubes | " | sixteen " | " |
| First four tubes | " | thirty-two " | " |
| All five tubes (lipid antigen) | " | 160 or more " | " |
| All five tubes, spinal fluid | " | 64 or more " | " |
| First five tubes (cardiolipin antigen) | | 64 Kolmer Units | " |
| First six tubes | " " | 128 " " | " |
| First seven tubes | " " | 256 or more " | " |

Kolmer Units are computed by the formula S = 4D, where S is the serum or spinal fluid potency and D is the highest dilution giving a positive reaction.

Below is a table indicating the method of reporting in "dils." Many civilian laboratories prefer the end point titer be reported in "dils" (a contraction of the word dilution) in reporting all quantitative tests for better correlation of results.

Table 22. Reporting Test Results in Dils.

| Serums or Spinal Fluid | | | | | | | Report |
|---|---|---|---|---|---|---|---|
| Undiluted | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | |
| 4 | 3 | 1 | - | - | - | - | Reactive, 1:4 dilution, or 4 dils (positive) |
| 4 | - | - | - | - | - | - | Reactive, (4+), undiluted, or 1 dil. (positive) |
| 4 | 4 | 3 | 2 | - | - | - | Reactive, 1:8 dilution, or 8 dils. (positive) |
| 4 | 4 | 4 | 4 | 4 | 1 | - | Reactive, 1:32 dilution, or 32 dils. (positive) |
| 3 | 1 | - | - | - | - | - | Reactive, 1:2 dilution, or 2 dils. (positive) |
| 1 | - | - | - | - | - | - | Reactive, (1+), undiluted, or 1 dil. (positive) |
| 2 | 1 | + - | - | - | - | - | Reactive, 1:2 dilution, or 2 dils. (positive) |
| + | - | - | - | - | - | - | Weakly reactive. (doubtful) |
| - | - | - | - | - | - | - | Nonreactive. (negative) |
| 4 | 4 | 4 | 4 | 4 | 4 | 4 | Reactive, 1:64 or more dilution or 64 or more dils (positive) |

Note: If a Reactive result is obtained with the highest dilution of the regular quantitative test, higher dilutions may be prepared and tested.

6. Report the results of the quantitative tests in accordance with the following table, when the full hour incubation at 37°C. is required.

Table 23. Quantitative Test Reporting
(After 1 hour secondary incubation at 37° C.)

| Test tube reading | | | | | | | Control tube reading | Report[1] |
|---|---|---|---|---|---|---|---|---|
| 4 | 4 | 4 | 4 | 4 | 2 | - | 4+ | Anticomplementary |
| 4 | 4 | 3 | - | - | - | - | 2+ | Reactive. (positive) |
| 4 | 4 | 1 | - | - | - | - | 1+ | Reactive. (positive) |
| 4 | 4 | 1 | - | - | - | - | ± | Reactive. (positive) |
| 3 | 2 | - | - | - | - | - | ± | Reactive. (positive) |
| 4 | 4 | 1 | - | - | - | - | 3+ | Weakly reactive. (doubtful) |
| 3 | 2 | - | - | - | - | - | 1+ | Weakly reactive. (doubtful) |
| 2 | 1 | - | - | - | - | - | ± | Weakly reactive. (doubtful) |
| 3 | - | - | - | - | - | - | ± | Reactive. (positive) |
| 1 | - | - | - | - | - | - | ± | Nonreactive. (negative) |
| 1 | - | - | - | - | - | - | 1+ | Nonreactive. (negative) |
| 2 | - | - | - | - | - | - | 1+ | Nonreactive. (negative) |
| 2 | - | - | - | - | - | - | 2+ | Nonreactive. (negative) |

[1]Quantitative designations of dilution end points or dils are omitted.

## Complement

When difficulties are experienced, they are usually first ascribed to defective hemolysin or antigen, but since both of these keep very well, they are rarely responsible. In some cases difficulties are due to the use of a complement too low in hemolytic activity and particularly in the case of a preserved complement. This is especially likely to be true during the hot months of the year and also when the serums of underweight or pregnant guinea pigs are used, as well as those previously employed in innoculation tests. Under these conditions anticomplementary reactions may occur with incomplete hemolysis of the antigen, serum, and spinal fluid controls. When the unit of complement is more than 0.5 ml. of 1:30 dilution in the regular tests, it should not be used.

Furthermore, while the complement may be satisfactory from the standpoint of hemolytic activity, it may be defective because it is supersensitive to what may be called the occult anticomplementary effects of antigen, serum, or spinal flud. Under these circumstances inhibition of hemolysis occurs in those tubes carrying antigen, while the antigen, serum, and spinal fluid controls show complete hemolysis with the danger of reporting falsely positive or nonspecific reactions. When the pretested complement or the pooled complement of a large number of guinea pigs is employed, the occult anticomplementary effects are exceptional occurrences. However, since they may happen in quantitative and qualitative spinal fluid tests, it is recommended that egg albumin be used routinely in conducting these tests, especially if the pretested complement is not being used.

## Prezone Reactions

Prezone reactions may occur in quantitative tests with serums, giving reactions like - - 2 3 4, with complete hemolysis of the serum control, but sometimes with incomplete hemolysis of the antigen control. They occur rather infrequently when the pretested or pooled complement is being used. These may be prevented by the use of 50 per cent egg albumin. Zonal reactions may occur if the normal anti-sheep cell agglutinins have not been absorbed from the patient's serum.

## Hemolysin

As previously stated, this is usually first suspected but is generally least likely to be the cause of difficulty, especially if the hemolysin has been previously found to be satisfactory. The unit of antisheep hemolysin in the regular tests should be at least 0.5 ml. of 1:4,000. Hemolysins of this and higher strength are so easy to prepare that it is a mistake to use weaker products. If the saline solution and complement are satisfactory, a good hemolysin is rarely responsible, even when shipped over long distances or kept in a refrigerator over months or even years.

## Red Cells

When blood is obtained from an abattoir, the red cells of occasional animals possessing increased resistance to serum hemolysis are almost always found. The cause of this phenomenon is unknown; fortunately it is rare. The remedy is to discard the red cells and secure a fresh supply of blood. When the corpuscles of preserved blood tend to become too fragile, it is advisable to use 0.9 per cent of saline instead of the usual 0.85 per cent in conducting the tests.

## Antigen

Provided no mistakes have occurred in dilution and dosage, antigen is very rarely the cause of trouble. When the antigen control shows incomplete hemolysis, it is almost surely due to some component of the hemolytic system, especially a complement supersensitive to antigen, in which case egg albumin may be used.

## Anticomplementary Serums and Spinal Fluids

Serums and spinal fluids may be found to be anticomplementary, as shown by incomplete hemolysis of the controls. After experience has been gained, some of these reactions may be safely read, as stated, but as a general rule it is much safer and wiser to repeat the tests with fresh serum or spinal fluid, especially in the case of those technicians lacking experience in complement fixation work. It is infinitely better to repeat the test than to run the slightest chance of error, especially the regrettable error of rendering a falsely positive reaction. Sometimes the majority of serums or spinal fluids of a day's work show incomplete hemolysis of the controls, but this trouble is not due to anticomplementary effects on their part but rather to the use of a defective supersensitive complement. Under these conditions the tests may have to be repeated and for this reason the unused portion of the serums and of spinal fluids should always be kept in the refrigerator until the test is completed, in case repetitions are required. If difficulties are due to thermostable anticomplementary substances in serums, the serums can usually be satisfactorily tested after preparation by Sach's method, including the use of egg albumin.

## Complement Fixation Tests for Other Diseases

Complement fixation tests, employing specific antigens, are used in the diagnosis of many diseases, including amebiasis, typhoid fever, glanders, typhus fever, trichinosis, psittacosis, echinococcus, malaria, filariasis, Rocky Mountain spotted fever, trypanosomiasis, yaws, and pinta.

## SECTION VI

## MAZZINI MICROFLOCCULATION TEST WITH SERUM

Note: The outline below is the Mazzini Microflocculation Test employing lipoidal antigen. This has proven of value in a battery of tests, although for routine procedures the Mazzini Test employing cardiolipin antigen may be more reliable.

### Equipment

A rotating machine, ring maker, slide holder, 30 ml. glass-stoppered round bottle, and a 5 ml. syringe are necessary.

### Reagents

Mazzini cholesterolized antigen and buffered 1 per cent saline solution, pH 6.3 to 6.4 (obtained with antigen) are used.

### Preparation of Serums

The identical procedure, which was used with the Kahn test, is followed.

### Preparation of Antigen Suspension

1. Pipet 3.0 ml. of buffered saline to the bottom of a 30 ml. bottle.

2. Measure 0.4 ml. of cholesterolized antigen (with a 1 ml. pipet graduated to the tip). For only a few tests, 1.5 ml. of buffered saline may be used with 0.2 ml. of antigen.

3. Hold the bottle in the left hand and impart a rapid rotating motion to it as the antigen is being discharged directly and at once into the saline solution from the pipet held in the right hand (antigen is blown from pipet).

4. Mix by drawing the suspension into the pipet and blowing it out two or three times.

5. Allow the suspension to "ripen" at room temperature in a dark place for 3 hours. (This suspension is usable during the working day.)

6. Shake the antigen suspension gently from the bottom to the top of the bottle several times.

7. Transfer the suspension to the 5 ml. syringe fitted with a 25 gage needle which has been previously calibrated to deliver 0.01 ml. of antigen suspension per drop, when held in a vertical position.

Performance of Test

1. To a paraffin-ringed glass slide pipet 0.05 ml. of previously inactivated serum.

2. To the serum add 1 drop of previously prepared antigen suspension.

3. Rotate for 4 minutes at 120 rpm by hand* or 180 rpm on Boerner type mechanical rotator.

4. Check for clumping under a low power microscope using 100 x magnification.

| | |
|---|---|
| No clumping ..... | Negative |
| Very small clumps..... | One plus |
| Small clumps ..... | Two plus |
| Medium clumps ..... | Three plus |
| Large clumps ..... | Four plus |

5. Add a second drop of antigen to each test that gives a 1+, 2+, or atypical reaction, rotate slide for 4 minutes, and make a second reading.

6. Examine microscopically. If the reading is stronger, record the result; if weaker, record the original result.

Note: Positive and negative controls are required.

7. Report results as follows:

| Readings | Report |
|---|---|
| Negative ..... | Nonreactive (Negative) |
| 1+ ..... | Weakly reactive (Doubtful) |
| 2+ ..... | Weakly reactive (Doubtful) |
| 3+ ..... | Reactive (Positive) |
| 4+ ..... | Reactive (Positive) |

*When rotating the slide by hand circumscribe a 2 inch circle.

## SECTION VII

## VDRL SLIDE FLOCCULATION TESTS WITH SERUM

### Equipment

1. Rotating machine, adjustable to 180 rpm, circumscribing a circle 3/4 inch in diameter on a horizontal plane.

2. Ringmaker, to make paraffin rings approximately 14 mm. in diameter.

3. Slide holder, for 2 x 3 inch microscope slides.

4. Hypodermic needles, of appropriate sizes, with or without points.

### Glassware

1. Slides,[1] 2 x 3 inch, with paraffin rings approximately 14 mm. in diameter.

2. Bottles,[2] 1 ounce, round, screw-capped (Vinylite or tinfoil-lined) or glass-stoppered, narrow mouth.

Note: Some of the 1 ounce, glass-stoppered bottles now available are unsatisfactory for preparing a single volume of antigen emulsion for these tests due to an inward bulging of the bottom that causes the 0.4 ml. of saline solution to be distributed only at the periphery. A satisfactory emulsion may be obtained if the 0.8 ml. of saline solution covers the bottom surface of this type of bottle when double quantities of antigen emulsion are prepared. Round bottles of approximately 35 mm. diameter with flat or concave inner-bottom surfaces are satisfactory for preparing single volumes of antigen emulsion.

The low cost of plastic caps recommends against attempts to clean these for reuse. The use of an unclean stopper or cap can be the cause of unsatisfactory emulsions.

3. Syringe, Luer-type, 1 or 2 ml.

---

[1]Glass slides with ceramic rings may also be used for the VDRL slide test with the following precautions. The rings must be high enough to prevent spillage when slides are rotated at prescribed speeds. Slides must be cleaned after each use so that serum will spread to the inner surface of the ceramic rings. This type of slide should be discarded if or when the ceramic ring begins to flake off, since these particles, in the test serums, may be mistaken for antigen particle clumps, thereby causing a false Reactive report.

[2]Catalog No. CA-1, 90525 (plain bottles); CA-1, 90530 (glass-stoppered bottles). Corning Glass Works, Corning, N.Y.

## Reagents

1. Antigen

a. Antigen for this test is an alcoholic solution containing 0.03 per cent cardiolipin, 0.9 per cent cholesterol, and sufficient purified lecithin to produce standard reactivity. During recent years this amount of lecithin has been 0.21 per cent $\pm$ 0.01 per cent.

b. Each lot of antigen must be serologically standardized by proper comparison with an antigen of known reactivity.

c. Antigen is dispensed in screw-capped (tinfoil or Vinylite liners) brown bottles or hermetically sealed glass ampules, and stored at room temperature (73° to 85°F.).

d. The components of this antigen remain in solution at normal temperature so any precipitate noted will indicate changes due to factors such as evaporation or additive materials contributed by pipets. Antigen containing precipitate should be discarded.

2. Saline solutions

a. Buffered saline solution containing 1 per cent sodium chloride:

| | |
|---|---|
| Formaldehyde, neutral, reagent grade, ml. ..... | 0.5 |
| Secondary sodium phosphate ($Na_2HPO_4 \cdot 12\ H_2O$), gm. ... | 0.093 |
| Primary potassium phosphate ($KH_2PO_4$), gm. ..... | 0.170 |
| Sodium chloride (A.C.S.), gm. ..... | 10.0 |
| Distilled water, ml. ..... | 1,000.0 |

This solution yields potentiometer readings of pH 6.0 $\pm$ 0.1 and is stored in screw-capped or glass-stoppered bottles.

b. 0.9 per cent saline solution:

Add 900 mg. of dry sodium chloride to each 100 ml. of distilled water.

## Preparation of Serum

1. Clear serum, obtained from centrifuged, clotted blood, is heated in a 56°C. water bath for 30 minutes before being tested.

2. All serums are examined when removed from the water bath and those found to contain particulate debris are recentrifuged.

3. Serums to be tested more than 4 hours after the original heating period should be reheated at 56°C. for 10 minutes.

Preparation of Slides

1. New slides are cleaned with Bon Ami which is removed with a soft cloth after drying.

2. Previously used slides are first freed of paraffin, washed with soap or detergent, rinsed free of cleaning compound, and then treated as new slides.

3. Slides are handled by the edges, while cleaning, to prevent greasy fingerprints on the testing surfaces.

4. Serums will spread within the circles on clean slides. Failure of the serums to spread is an indication that the slide is unclean and therefore should not be used.

5. Paraffin rings are made by transferring heated paraffin to the slides by means of metal molds.

Note: Glass slides with concavities or glass rings are not recommended for this test.

Preparation of Antigen Emulsion

1. Pipet 0.4 ml. of buffered saline solution to the bottom of a 1 ounce, round, glass or screw cap stoppered bottle.

2. Add 0.5 ml. of antigen (from the lower half of a 1.0 ml. pipet graduated to the tip) directly onto the saline solution while continuously but gently rotating the bottle on a flat surface.

Temperature of buffered saline solution and antigen should be in the range of 23° to 29°C. at time antigen emulsion is prepared.

Note: Antigen is added drop by drop, but rapidly, so that approximately 6 seconds are allowed for each 0.5 ml. of antigen. Pipet tip should remain in upper third of bottle and rotation should not be vigorous enough to splash saline solution on the pipet. Proper speed of rotation is obtained when the outer edge of the bottle circumscribes a 2 inch diameter circle approximately three times per second.

3. Blow last drop of antigen from pipet without touching pipet to saline solution.

4. Continue rotation of bottle for 10 seconds more.

5. Add 4.1 ml. of buffered saline solution from 5 ml. pipet.

6. Place top on bottle and shake vigorously for approximately 10 seconds.

7. Antigen emulsion is then ready for use and may be used during 1 day.

Double this amount of antigen emulsion may be prepared at one time by using doubled quantities of antigen and saline solution. A 10 ml. pipet should then be used for delivering the 8.2 ml. volume of saline solution. If larger quantities of antigen emulsion are required, more than one mixture should be prepared. These aliquots may then be tested and pooled.

Testing Antigen-Emulsion Delivery Needles

1. The number of antigen particles per microscopic field is determined by the size of antigen-emulsion drop used. For this reason the needle used each day should be checked.

2. Antigen emulsion is dispensed from a syringe fitted with a 22 gage, regular-bevel, a 23 gage, long-bevel hypodermic needle, or an 18 gage needle without a point.

Note: It is of primary importance that the proper amount (1/60 ml.) of antigen emulsion be used in each qualitative test, so any method of delivery that will produce drops of constant and proper size is adequate. (See Fig. 2, page 72.)

3. If the four-fifths volume quantitative VDRL slide method (method B) is employed, a smaller gage needle (with or without point) that will deliver 75 drops of antigen emulsion per milliliter must be employed for this procedure. A 19 gage needle, without point, is used at the Venereal Disease Research Laboratory. Practice will allow rapid delivery of antigen emulsion but care should be exercised to obtain drops of constant size.

4. When allowed to stand, antigen emulsion should be gently mixed before use by rotating the bottle and emptying and refilling the syringe.

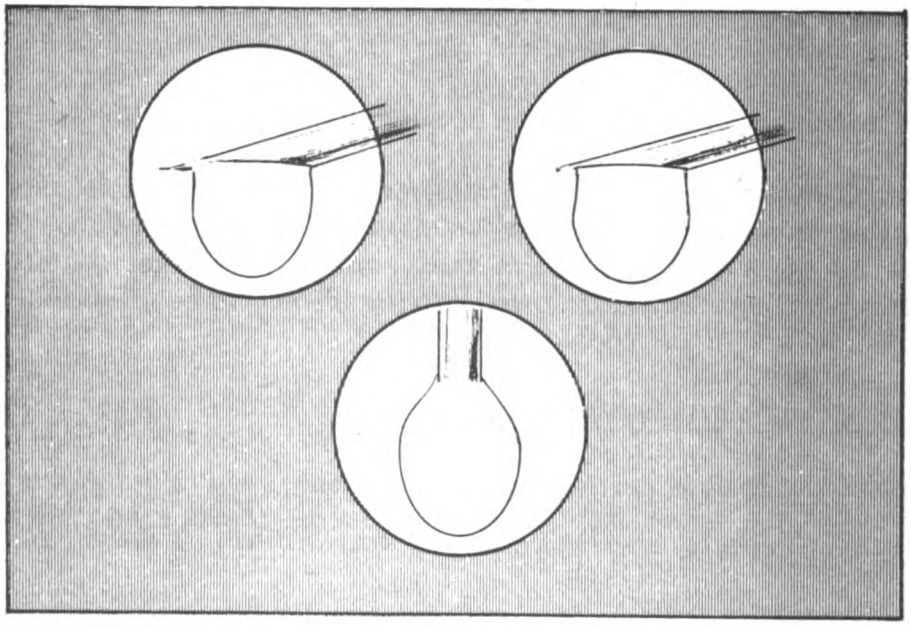


Fig. 2. Same Size Drop

Preliminary Testing of Antigen Emulsion

1. Each preparation of antigen emulsion should first be examined by testing serums of known reactivity in the Reactive, Weakly Reactive, and Nonreactive zones. This is accomplished by the method described under "VDRL Slide Qualitative Test With Serum." These tests should present typical results and the size and number of antigen particles in the Nonreactive serum should be optimum.

2. Only those antigen emulsions that have produced the designated reactions in tests performed with control serums (Reactive, Weakly Reactive, and Nonreactive) should be used. If antigen particles in the Nonreactive serum tests are too large, the fault may be in the manner of preparing antigen emulsion, although other factors may be responsible.

3. An unsatisfactory antigen emulsion should not be used.

VDRL Slide Qualitative Test With Serum

1. Pipet 0.05 ml. of heated serum into one ring of a paraffin-ringed glass slide.

2. Add one drop (1/60 ml.) of antigen emulsion onto each serum.

3. Rotate slides for 4 minutes. (Mechanical rotators that circumscribe a

3/4 inch diameter circle should be set at 180 rpm. Rotation by hand should circumscribe a 2 inch diameter circle 120 times per minute.)

4. Read tests immediately after rotation.

Note: Serum controls of graded reactivity (Reactive, Weakly Reactive, and Nonreactive) are always included during a testing period to insure proper reactivity of antigen emulsion at time tests are run.

## Reading and Reporting Slide Qualitative Test Results

1. Read tests microscopically, with low power objective, at 100 x magnification. The antigen particles appear as short rod forms at this magnification.

Aggregation of these particles into large or small clumps is interpreted as degrees of reactivity.

| Reading | Report |
|---|---|
| No clumping or very slight roughness ..... | Nonreactive (N). |
| Small clumps ..... | Weakly Reactive (WR). |
| Medium and large clumps ..... | Reactive (R). |

2. Zonal reactions, due to an excess of Reactive serum component, are recognized by irregular clumping and the loosely bound characteristics of the clumps. The usual Reactive finding is characterized by large or small clumps of fairly uniform size. Experience will allow differentiation to be made between this type of reaction and the zonal picture wherein large and/or small clumps may be intermingled with free antigen particles. A zonal reaction is reported as Reactive. In some instances, this zoning effect may be so pronounced that a Weakly Reactive result is produced by a very strongly Reactive serum. It is therefore recommended that all serums producing Weakly Reactive results in the qualitative test be retested using the quantitative procedure before a report of the VDRL slide test is submitted. When a Reactive result is obtained on some dilution of a serum that produced only a Weakly Reactive result as undiluted serum, the report is Reactive (see "Reading and Reporting Slide Quantitative Test Results," under "VDRL Slide Quantitative Tests With Serum").

## VDRL Slide Quantitative Tests With Serum

All serums that produce Reactive or Weakly Reactive results in the qualitative VDRL slide test should be quantitatively retested by one of the two methods referred to as quantitative tests A or B. Since both of these procedures, in most instances, provide for direct measurements of serum and saline solution, either method is efficient in its requirement of technician-time and amount of glassware employed. Since

quantitative test A uses serum dilutions of 1:2.5, 1:5, 1:10, etc., the alternate quantitative test B has been added for those laboratories desiring the doubling, serum-dilution scheme of 1:2, 1:4, 1:8, 1:16, etc.

VDRL Slide Quantitative Test A

1. Place four 2 x 3 inch glass slides containing twelve 14 mm. paraffin rings in a 5 place slide holder.

2. Place a glass slide with two parallel strips of masking or adhesive tape in the center space of the slide holder.

Numbers identifying the serums to be tested (four on the two slides above the numbered slide and four on the two lower slides) are written on the adhesive strips.

3. Prepare a 1:10 dilution of each serum to be tested quantitatively by adding 0.1 ml. of the heated serum to 0.9 ml. of 0.9 per cent saline solution using a 0.2 ml. pipet graduated in 0.01 ml.

4. Mix the serum and saline solution thoroughly and then allow the pipet to stand in the test tube.

5. Using this 0.2 ml. pipet, transfer 0.05 ml., 0.02 ml., and 0.01 ml. quantities of the 1:10 dilution of the first serum into the fourth, fifth, and sixth rings, respectively.

6. With the same pipet, transfer 0.05 ml., 0.02 ml., and 0.01 ml. quantities of the first serum, undiluted, into the first, second, and third ringed areas, as illustrated on page 76.

7. Repeat this procedure with each serum and the accompanying 1:10 serum dilution until each of the eight serums are pipetted onto the slides.

8. Add one drop (0.03 ml.) of 0.9 per cent saline solution to the second and fifth rings of each serum, by vertical delivery, from a 15 gage hypodermic needle fitted to a glass syringe.

9. Add one drop (0.04 ml.) of 0.9 per cent saline solution to the third and sixth rings of all eight serums by vertical delivery from the syringe fitted with the 13 gage needle. The six mixtures of each serum are then equivalent to dilutions of 1:1 (undiluted), 1:2.5, 1:5, 1:10, 1:25, and 1:50.

10. Rotate slides gently by hand for about 15 seconds to mix the serum and saline solution.

11. Add one drop (1/60 ml.) of antigen emulsion to each ring using a syringe and needle as prescribed in the technique for the slide qualitative serum test.

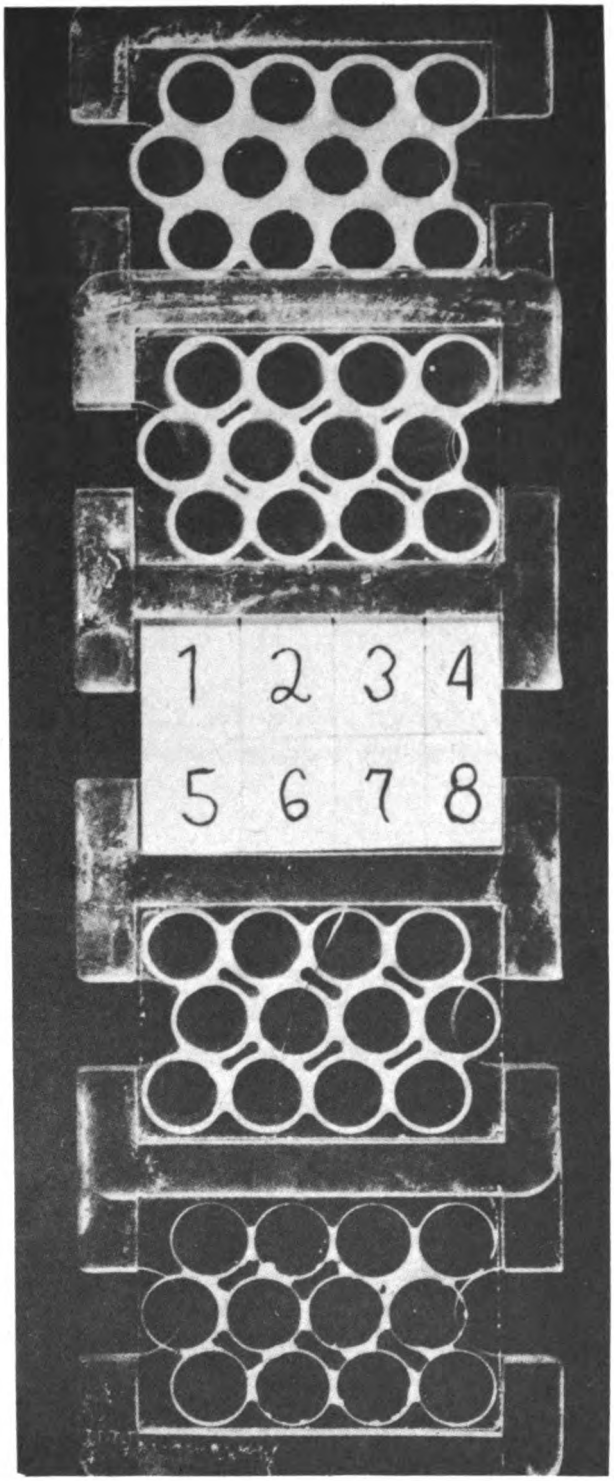
This manner of preparing serum dilutions by adding serum and saline solution directly to the slides is outlined in Quantitative Test B, page 77.

12. Complete tests by rotation of the slides in the manner prescribed for the "VDRL Slide Qualitative Test With Serum."

13. Read results microscopically. The highest serum dilution giving a Reactive result (not Weakly Reactive) is reported as the reactivity end point of the serum, e.g., Reactive — 1:25 dilution, or, Reactive — 25 dils.

14. If all serum dilutions tested give Reactive results, prepare a 1:100 dilution of that serum by diluting 0.1 ml. of the 1:10 serum dilution with 0.9 ml. of 0.9 percent saline solution.

15. Pipet 0.05 ml., 0.02 ml., and 0.01 ml. amounts of this 1:100 serum dilution onto each ring and add enough saline solution to bring the volumes to 0.05 ml. Serum dilutions of 1:100, 1:250, and 1:500 are thus prepared. Test these dilutions of serum exactly as the lower dilutions are tested.




| Ring No. | Quantitative Test A | | | Quantitative Test B | | |
|---|---|---|---|---|---|---|
| | Serum | Saline solution | Serum dilutions | Serum | Saline solution | Serum dilutions |
| | (ml.) | (ml.) | | (ml.) | (ml.) | |
| 1 | 0.05 | 0 | 1:1 (undiluted) | 0.04 | 0 | 1:1 (undiluted) |
| 2 | .02 | .03 | 1:2.5 | .02 | .02 | 1:2 |
| 3 | .01 | .04 | 1:5 | .01 | .03 | 1:4 |
| 4 | (diluted 1:10) .05 | 0 | 1:10 | (diluted 1:8) .04 | 0 | 1:8 |
| 5 | .02 | .03 | 1:25 | .02 | .02 | 1:16 |
| 6 | .01 | .04 | 1:50 | .01 | .03 | 1:32 |
| | Serum numbers | | | | | |
| 1 | .05 | 0 | 1:1 | .04 | 0 | 1:1 |
| 2 | .02 | .03 | 1:2.5 | .02 | .02 | 1:2 |
| 3 | .01 | .04 | 1:5 | .01 | .03 | 1:4 |
| 4 | (diluted 1:10) .05 | 0 | 1:10 | (diluted 1:8) .04 | 0 | 1:8 |
| 5 | .02 | .03 | 1:25 | .02 | .02 | 1:16 |
| 6 | .01 | .04 | 1:50 | .01 | .03 | 1:32 |

**Fig. 3. Slides and Slide Holder for Quan. Tests**

## VDRL Slide Quantitative Test B

1. Place four 2 x 3 inch glass slides with 12 paraffin rings in a 5 place slide holder (see Fig. 3 ), with a numbered slide in the center space exactly as described for "Slide Quantitative Test A."

2. Prepare a 1:8 dilution of each serum by adding 0.1 ml. of the heated serum to 0.7 ml. of the 0.9 per cent saline solution using a 0.2 ml. pipet graduated in 0.01 ml.

3. Mix the serum and saline solution thoroughly and then allow the pipet to stand in the test tube.

4. Using this pipet, transfer 0.04 ml., 0.02 ml., and 0.01 ml. quantities of the 1:8 serum dilution into the fourth, fifth, and sixth paraffin rings, respectively.

5. With the same pipet, transfer 0.04 ml., 0.02 ml., and 0.01 ml. of the undiluted serum into the first, second, and third paraffin rings, respectively.

6. Repeat this procedure with each serum and the accompanying 1:8 serum dilution until each of the eight serums are pipetted into their respectively numbered places on the slides.

7. Add two drops (0.01 ml. in each drop) of 0.9 per cent saline solution to the second and fifth rings of each serum, by vertical delivery from a 23 gage[3] hypodermic needle fitted to a glass syringe.

8. Add three drops of 0.9 per cent saline solution (delivered in the same manner) of the same size to the third and sixth rings of each serum.

9. Rotate slides gently by hand for about 15 seconds to mix the serum and saline solution.

10. Add one drop (1/75 ml.) of antigen emulsion to each ring using a syringe and needle of appropriate size. (Caution. --Note that the amount of antigen emulsion used in this method has been reduced to 1/75 ml. to correspond with the reduced serum volume of 0.04 ml.)

11. Complete tests in the manner described for the "VDRL Slide Qualitative Test With Serum" (p. 72) and read results microscopically immediately after rotation.

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[3] Needles should be checked for proper drop size. Saline solutions may be delivered from a 19 gage needle (0.02 ml. per drop) and a 15 gage needle (0.03 ml. per drop).

By this method, the dilutions of each serum are 1:1 (undiluted), 1:2, 1:4, 1:8, 1:16, and 1:32.

12. If all serum dilutions tested produce Reactive results, prepare a 1:64 dilution of that serum in saline solution. Add seven parts of saline solution to one part of the 1:8 serum dilution, and test in three amounts as was done with the 1:8 serum dilutions. Dilutions prepared from the 1:64 dilution will be equivalent to 1:64, 1:128, and 1:256.

Reading and Reporting Slide Quantitative Test Results

1. Read tests microscopically at 100 X magnification as described for the qualitative procedure.

2. Report results in terms of the greatest serum dilution that produces a Reactive (not Weakly Reactive) result in accordance with the following examples:

Method A

| Undiluted serum | Serum dilutions | | | | Report |
|---|---|---|---|---|---|
| 1:1 | 1:2.5 | 1:5 | 1:10 | 1:25 | |
| R | WR | N | N | N | Reactive, undiluted only, or 1 dil. |
| R | R | WR | N | N | Reactive, 1:2.5 dilution, or 2.5 dils. |
| R | R | R | WR | N | Reactive, 1:5 dilution, or 5 dils. |

Method B

| Undiluted serum | Serum dilutions | | | | Report |
|---|---|---|---|---|---|
| 1:1 | 1:2 | 1:4 | 1:8 | 1:16 | |
| R | WR | N | N | N | Reactive, undiluted only, or 1 dil. |
| R | R | WR | N | N | Reactive, 1:2 dilution, or 2 dils. |
| R | R | R | WR | N | Reactive, 1:4 dilution, or 4 dils. |

R = Reactive. WR = Weakly Reactive N = Nonreactive

Note: Under conditions of high temperature and low humidity which are sometimes present during the summer months in certain areas, antigen emulsion may be stored in the refrigerator but should be restored to room temperature before use. To avoid surface drying under these conditions, tests should be completed and read as rapidly as possible. Slide covers containing a moistened blotter may be employed.

## VDRL Tube Flocculation Tests with Serum

### Equipment

1. Kahn shaking machine (must be operated at 275 to 285 oscillations per minute).

2. Reading lamp, fluorescent or gooseneck-type.

### Reagents

1. Antigen. (VDRL slide flocculation test antigen).

2. Saline solutions.
   a. 1 per cent buffered saline solution. (Prepare as for the VDRL slide flocculation tests).
   b. Unbuffered 1 per cent sodium chloride solution.
   Add 1 gm. of dry sodium chloride (A.C.S.) to each 100 ml. of distilled water.

### Preparation of Serum

1. Clear serum, removed from centrifuged, clotted, whole blood, is heated in a 56° C. water bath for 30 minutes before being tested.

2. All serums are examined when removed from the water bath and those found to contain particulate debris are recentrifuged.

3. Serums to be tested more than 4 hours after being heated should be reheated at 56° C. for 10 minutes.

### Preparation of Antigen Emulsion

1. Prepare antigen emulsion as described for the VDRL slide flocculation tests.

2. Add four parts of 1 per cent sodium chloride solution to one part of VDRL slide test emulsion. Mix well and allow to stand five or more minutes (not longer than 2 hours) before use. This solution will be referred to as "diluted antigen emulsion." Resuspend diluted antigen emulsion before use.

### VDRL Tube Qualitative Test with Serum

1. Pipet 0.5 ml. of heated serum into a 12 x 75 mm. (outside dimension) test tube.
2. Add 0.5 ml. of diluted antigen emulsion to each serum.

3. Shake tubes on Kahn shaker for 5 minutes.

4. Centrifuge all tubes for 10 minutes at force equivalent to 2,000 rpm in No. 1 or 1,700 rpm in No. 2 I.E.C.[4] centrifuge with horizontal heads.

5. Return tubes to the Kahn shaking machine and shake for exactly 1 minute.

Note: Include Reactive and Nonreactive control serums in each test run.

Reading and Reporting Tube Qualitative Test Results

1. Read test results as soon as secondary shaking period is completed by holding tubes close to the shade of a reading lamp with a black background, at approximately eye level. A shaded fluorescent desk lamp or a gooseneck-type lamp with a blue bulb is a satisfactory reading light source.

2. Record results as follows:

Reactive ..... Visible aggregates in a clear or slightly turbid medium. All borderline reactions, where the observer has doubt regarding visible clumping should be reported as Nonreactive.

Nonreactive ..... No visible clumping or aggregation of antigen particles. Appearance slightly turbid or granular. Definite silken swirl on gentle shaking.

Note: Turbid or hemolyzed serums may cause completed tests to be too turbid for macroscopic reading and are therefore unsatisfactory specimens for this test.

Zonal Reactions, due to excess of Reactive serum component, may appear to be very weak or, in rare instances, Nonreactive. Whenever a zonal reaction is suspected, another test should be performed using 0.1 ml. of heated serum and 0.4 ml. of saline solution in place of the original 0.5 ml. of serum. If a Reactive finding is obtained with the smaller amount of serum, a Reactive report should be issued.

VDRL Tube Quantitative Test with Serum

1. Pipet 0.5 ml. of freshly prepared 0.9 per cent saline solution into each of five or more test tubes (12 x 75 mm.) omitting the first tube.

2. Add 0.5 ml. of heated serum to the first and second tubes. (The first tube may be omitted if the VDRL tube qualitative test has been performed, and if sufficient serum is not available.)

3. Mix and transfer 0.5 ml. from second to third tube.

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[4]International Equipment Co., Boston, Mass.

4. Continue mixing and transferring 0.5 ml. from each tube to the next until the last tube is reached.

5. Mix and discard 0.5 ml. from last tube.

6. Add 0.5 ml. of diluted antigen emulsion to each tube and proceed as described under "VDRL Tube Qualitative Test with Serum."

## Reading and Reporting Tube Quantitative Test Results

The greatest serum dilution producing a definitely Reactive result is reported as the reactivity end point as shown in the following examples:

Table 1. Reporting Results

| Undiluted serum | Serum dilutions | | | | Report |
|---|---|---|---|---|---|
| 1:1 | 1:2 | 1:4 | 1:8 | 1:16 | |
| R | N | N | N | N | Reactive, undiluted only, or 1 dil. |
| R | R | R | N | N | Reactive, 1:4 dilution, or 4 dils. |
| R | R | R | R | N | Reactive, 1:8 dilution, or 8 dils. |
| R = Reactive. N = Nonreactive | | | | | |

## VDRL Tests with Spinal Fluid

### Equipment

1. Kahn shaking machine (must be operated at 275 or 285 oscillations per minute).

### Reagents

1. Antigen (VDRL slide flocculation test antigen.)

2. Saline solutions

a. 1 per cent buffered saline solution. (Prepare as for the VDRL slide flocculation tests.)

b. 10 per cent sodium chloride solution.
Dissolve 10 gm. of dry sodium chloride (A.C.S.) in 100 ml. of distilled water.

### Preparation of Spinal Fluid

1. Centrifuge and decant each spinal fluid. Spinal fluids which are visibly contaminated or contain gross blood are unsatisfactory for testing.

2. Heat spinal fluid at 56° C. for 15 minutes. Cool to room temperature before testing.

## Preparation of the Sensitized Antigen Emulsion

1. Prepare antigen emulsion as described for the VDRL slide flocculation tests (see "Preparation of Antigen Emulsion", see page 70.)

2. Add one part of 10 per cent sodium chloride solution to one part of VDRL slide test emulsion.

3. Mix well, and allow to stand at least 5 minutes but not more than 2 hours before use.

## VDRL Qualitative Test with Spinal Fluid

1. Pipet 1.0 ml. of heated spinal fluid into a 13 x 100 mm. test tube. Include Reactive and Nonreactive spinal fluid controls with each series of tests run.

2. Add 0.2 ml. of sensitized antigen emulsion to each spinal fluid.
Resuspend the sensitized antigen emulsion immediately before use by inverting container several times.

3. Shake racks of tubes on Kahn shaking machine for 15 minutes.

4. Centrifuge all tubes for 5 minutes at force equivalent to 1,800 rpm in No. 1 or 1,600 rpm in No. 2 I.E.C.[4] centrifuge.

5. Return tubes to Kahn shaking machine and shake exactly 2 minutes.

## Reading and Reporting Qualitative Test Results

1. Read test results as soon as possible after the secondary shaking period by holding tubes close to the shade of a desk lamp having a black background.

Note: Each tube may be held motionless or shaken gently during the reading. Excessive agitation should be avoided.

2. Report results as follows:

| | |
|---|---|
| Reactive ..... | Definitely visible aggregates suspended in a water-clear or turbid medium. |
| Nonreactive ..... | No aggregation, complete dispersion of particles, appearance turbid or slightly granular. |

[4]International Equipment Co., Boston, Mass.

## VDRL Quantitative Test with Spinal Fluid

Quantitative tests are performed on all spinal fluids found to be Reactive in the qualitative test.

1. Prepare spinal fluid dilutions as follows:

a. Pipet 1.0 ml. of 0.9 per cent sodium chloride solution into each of five or more tubes.

b. Add 1.0 ml. of heated spinal fluid to tube 1, mix well, and transfer 1.0 ml. to tube 2.

c. Continue mixing and transferring from one tube to the next until the last tube contains 2 ml. Discard 1.0 ml. from the last tube. The respective dilution ratios are 1:2, 1:4, 1:8, 1:16, 1:32, etc.

2. Test each spinal fluid dilution as described under "VDRL Qualitative Test with Spinal Fluid."

## Reading and Reporting Quantitative Test Results

1. Read each tube as described under "VDRL Qualitative Test with Spinal Fluid."

2. Report test results in terms of the highest dilution of spinal fluid producing a Reactive result. The term "dils" which expresses the same dilution reactivity end point may be applied.

Example:

Table 2. Reporting Results

| Spinal fluid dilutions | | | | | Report |
|---|---|---|---|---|---|
| 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | |
| N | N | N | N | N | Reactive,[1] undiluted only, or 1 dil. |
| R | R | R | N | N | Reactive, 1:8 dilution, or 8 dils. |
| R | R | R | R | N | Reactive, 1:16 dilution, or 16 dils. |

R = Reactive. N = Nonreactive.

[1] Reactive finding with undiluted spinal fluid in the qualitative test.

## References

(1) Harris, A.; Rosenberg, A.A.; Riedel, L.M.: A microflocculation test for syphilis using cardiolipin antigen. Preliminary report. J. Ven. Dis. Inform., 27: 169-174, July 1946.

(2) Harris, A.; Rosenberg, A.A.; Del Vecchio, E.R.: The VDRL slide flocculation test for syphilis. II. A supplementary report. J. Ven Dis. Inform., 29: 72-75, March 1948.

(3) Harris, A.; Rosenberg, A.A.; Del Vecchio, E.R.: A macroflocculation test for syphilis using cardiolipin-lecithin antigen. J. Ven. Dis. Inform., 29: 313-316, October 1948.

(4) Rosenberg, A.A.; Harris, A.; Harding, V.L.: A macroflocculation spinal fluid test employing cardiolipin-lecithin antigen. J. Ven. Dis. Inform., 29: 359-361, December 1948.

## SECTION VIII

## THE MICROFLOCCULATION TEST (CARDIOLIPIN) EMPLOYED BY THE ARMY AND AIR FORCE

The cardiolipin slide microflocculation test used by the Army, Air Force and Veteran's Administration differs from the VDRL in:

A. The amount of lecithin used in the antigen.
B. The slide used plus the method of cleaning the slides.
C. It is not to be converted to a tube test.

It is of utmost importance that with either antigen, the technic be adhered to as outlined.

### Materials and Methods

#### Antigen

Antigen for the test contains cardiolipin 0.03 per cent, purified lecithin 0.27 per cent, and cholesterol 0.90 per cent in ethyl alcohol (1). This standard antigen (2) is supplied by the Army Medical Service Graduate School, Washington 12, D.C. No other preparation will be employed.

#### Antigen diluent

The antigen diluent is a phosphate buffered 1.0 per cent solution of sodium chloride having a pH of 6.0 ± 0.1. It is prepared as follows by dissolving the given reagents in distilled water sufficient to give a final volume of one liter. The pH of the final solution is checked potentiometrically (Beckman pH meter). Antigen diluent (3) also is supplied by the Army Medical Service Graduate School, Washington 12, D.C.

| | |
|---|---|
| Sodium chloride, (A.C.S.) ..... | 10.0 gms. |
| Sodium monohydrogen phosphate ..... ($Na_2HPO_4 + 12H_2O$) | 0.093 gms. |
| Potassium dihydrogen phosphate ..... ($KH_2PO_4$) | 0.170 gms. |
| Formaldehyde solution (37 per cent) ... neutral, reagent grade | 0.50 ml. |
| Distilled water sufficient to make | 1000 ml. |

### Serum

Clear serum obtained by centrifuging whole clotted blood is heated for 30

minutes in the water bath at 56° C. before testing. All serums are examined upon removal from the water bath, and those found to contain particulate material are clarified by recentrifugation. Serums to be tested more than four hours after heating should be reheated for 10 minutes in the water bath at 56° C.

Slides

Glass slides (A. H. Thomas Co., Specification, 7049-M) are used. These are 2 1/4 x 4 1/4 inch slides each with 10 mould-pressed cells in two rows of 5 each. New slides are first cleaned with sulfuric and chromic acid mixture (4b). They are then rinsed repeatedly in hot running tap water to remove all traces of the cleaning solution, and are rinsed finally in distilled water. Used slides are immersed in a pan of tap water immediately after the tests have been read in order to prevent drying of the serum-antigen mixture. As soon as possible they are washed carefully with soap and water, using a soft bristle brush, and are then freed of soap by repeated rinsings with hot tap water and distilled water as above. All slides in continual use should be cleaned at least once weekly with the sulfuric and chromic acid mixture. Bon Ami should not be used since it is difficult to remove from the moats surrounding the cells.

Cleaned slides are inverted and allowed to drain on a clean hand towel. They may then be wiped dry with a clean soft cloth, and are stored on edge in a clean, dust-proof box. Slides are handled by the edges while cleaning to prevent greasy finger prints on the testing surfaces. Serum will spread within the cells on clean slides. Failure to spread indicates that the slide is unclean and should not be used.

Preparation of antigen emulsion

Antigen emulsion is prepared by pipetting 0.4 ml. of the buffered antigen diluent to the bottom of a 30 ml. round glass bottle fitted with a glass or screw cap stopper. While the bottle is being rotated gently and continuously on a flat surface, 0.5 ml. of the antigen is added drop by drop (rapidly, without long intervals between drops) from the lower half of a 1.0 ml. pipet that is graduated to the tip. During this operation the pipet tip should remain in the upper third of the mixing bottle, antigen should be dropped directly into the diluent, and rotation should not be so vigorous as to splash diluent on the pipet tip. The last drop of antigen is blown from the pipet without touching it to the diluent, and rotation is continued for 10 seconds more. Using a 5.0 ml. pipet, 4.1 ml. of antigen diluent is than added, the top is placed on the bottle, and the latter is shaken vigorously for 10 seconds (throwing the liquid from the bottom to the top of the bottle). The antigen emulsion is then ready for use and may be used during one day if stored at refrigerator or room temperature. This amount of antigen (5.0 ml.) is sufficient for approximately 250 qualitative serum tests.

Twice this amount of antigen emulsion may be prepared at one time in a 30 ml. bottle by using doubled quantities of antigen and antigen diluent. A 10.0 ml. pipet

should then be used for delivering the required 8.2 ml. of diluent. If larger quantities of antigen are required, more than one mixture should be prepared and the emulsions then pooled and tested. (See pretesting of antigen emulsion).

Note: Under conditions of high temperature and low humidity such as are encountered during the summer months in certain areas, antigen emulsion may be stored in the refrigerator. To minimize the effects of evaporation in tests carried out under these conditions, the tests should be completed and read as rapidly as possible.

## Preliminary Standardization Procedures

### Pretesting of antigen delivery needle

Since the number of antigen particles per microscopic field is dependent upon the size of the emulsion drop, the antigen delivery needle should be checked each day of use.

Antigen emulsion is delivered from a 23 gage, long bevel, hypodermic needle attached to a 1.0 or 2.0 ml. syringe, which is allowed to stand in the antigen emulsion bottle when not in use. About 60 drops should be obtained from one ml. of antigen emulsion. This can be accomplished by holding the syringe so that the needle bevel faces downward and its dropping surface is horizontal. Increasing the angle at which the syringe is held decreases the dropping surface and consequently reduces drop size. Practice will allow rapid delivery of antigen emulsion, but care must be taken to obtain drops of equal size. When the antigen emulsion has been allowed to stand, it should be gently mixed before use by rotating the bottle and by filling and emptying the syringe.

### Pretesting of antigen emulsion

Each preparation of antigen emulsion should first be examined in preliminary tests of known reactive and non-reactive control serums. The former should be tested as indicated under the Quantitative Test, the latter as indicated under the Qualitative Test. Clumping in the case of reactive serum should be typical and of standard degree; the tests with non-reactive serum should show the optimal number of antigen particles per microscopic field.

## The Qualitative Test

### Technique

1. Pipet 0.05 ml. each of ten clear heated serums into the corresponding ten rings on a glass slide.

2. Add one drop (approximately 1/60th ml.) of antigen emulsion to each 0.05 ml. of serum.

3. Rotate the slides for four minutes at 180 rotations per minute using a Boerner type electric rotating machine. (If it is ever necessary to resort to hand rotation, this should be performed on a smooth flat surface at a rate of 120 rotations per minute; the movement should circumscribe a circle approximately two inches in diameter.)

4. Read the tests immediately after rotation.

Note: Always include as controls tests of known reactive and non-reactive serums, testing the former as indicated under the quantitative test, the latter as indicated under the qualitative test.

Reading and Reporting Results

Proper reading of test results requires the technician to have had adequate training and experience with the given techniques.

The tests are read microscopically with low power objective at 100 x magnification. The antigen particles in properly prepared emulsions appear as short rod forms. Aggregation of the particles into small, medium, or large clumps is translated into degree of reaction and the results recorded as follows:

Table 1. Reading and Reporting Results

| Microscopic appearance | Degree of reaction | Results |
|---|---|---|
| No clumping or very slight roughness | - | No reaction (-) |
| Small clumps | $\pm$ or 1 + | Weak reaction (WR) |
| Medium or large clumps | 2 +, 3 +, or 4 + | Reaction (R) |

Occasional serums may exhibit a zonal (atypical) type of reaction; i.e., show an apparently weak reaction ($\pm$ to 1+) in the qualitative test, but react to higher degree when retested in dilution (quantitative test). Zonal reactions may be recognized by atypical or feathery clumping and by the loosely bound characteristics of the clumps. The usual type of reaction is characterized by clumps of fairly uniform size, and experience will facilitate the differentiation of this ordinary type of reaction from zonal reactions, in which clumps of varying size are intermingled with free antigen particles. Whenever a zonal reaction is observed or suspected, the serum should be subjected to the quantitative test.

The Quantitative Test

Technic

Quantitative tests are performed with serial dilutions of serum in saline solution. Each dilution is treated as an individual serum and is tested as in the

qualitative test. Serial two-fold dilutions of serum are prepared by measuring 0.5 ml. of freshly prepared 0.9 per cent sodium chloride solution into each of 6 or more test tubes. 0.5 ml. of heated serum is added to tube 1, mixed well, and 0.5 ml. of the diluted serum transferred to tube 2. This operation is continued through the successive tubes until tube 6 contains 1.0 ml. of diluted serum. The resulting serum dilutions are: 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64. Each dilution is tested as in the qualitative test. In the occasional instance when a reaction is obtained with the 1:64 dilution, 0.5 ml. of this dilution (tube 6) is used to prepare an additional series of serum dilutions by transferring and mixing with successive 0.5 ml. volumes of saline solution.

Reporting results

The following are examples of the types of serologic result encountered; the methods used in recording and reporting are indicated. Results are reported as reaction (2+, 3+, 4+), weak reaction (±, 1+), or no reaction (-). Titer, in the case of reactive serums, is reported in terms of the highest dilution exhibiting a reaction (2+ to 4+).

Table 2. Reporting Results

| Serum | Qualitative test | Quantitative Test | | | | | | | | Result* and titer |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Results with serum dilutions | | | | | | | | |
| | | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | |
| A | 4 | 3 | 1 | - | - | - | - | - | - | R (2) |
| B | - | Test not required | | | | | | | | - |
| C | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 2 | 1 | R (128) |
| D | 2 | - | - | - | - | - | - | - | - | R (1) |
| E | 1 | Test not required | | | | | | | | WR |
| F | 1** | 3 | 4 | 4 | 2 | - | - | - | - | R (16) |

* Reaction (R), weak reaction (WR), or no reaction (-).
** Zonal reaction.

## REFERENCES


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2. Syphilis Antigen, Cardiolipin Microflocculation Test, 30 ml. Federal Stock No. 6505-261-7265.
3. Antigen Diluent, Buffered, Microflocculation Syphilis Test, 120 ml. Federal Stock No. 6505-158-6000.
4. Dept. of the Army Technical Manual, TM 8-227, Dept. of the Air Force Manual. AFM 160-14. 1951. (a) Serodiagnosis of syphilis, Chapter 12, 570-574. (b) Sulfuric and chromic acid mixture, Chapter 1, 10.

## SECTION IX

## CEREBROSPINAL FLUIDS

Cerebrospinal fluid is normally clear and colorless; it does not show clot formation on standing. Spinal fluids from healthy persons will show, in the great majority of cases, no cells, some will show two to three cells per cubic millimeter, and rarely, if ever, will one show more than five cells per cubic millimeter. The presence of more than five cells per cubic millimeter then can be looked upon as abnormal. All counts between 5 and 10 per cubic millimeter nearly always indicate disease, and counts above 10 per cubic millimeter are practically pathognomonic of disease of the brain or its meninges. Cell counts should be done as soon as possible after collection of the fluid, since the cells rapidly disintegrate, especially in turbid or purulent fluids. The cell count is always done on the second tube of spinal fluid collected which should be free of blood. Fair accuracy may often be had on cell counts ranging between 10 and 200 even after 24 or 48 hours, provided the cells have not been enmeshed in a clot and the fluid has been protected from contamination and kept in a refrigerator. Normal lumbar cerebrospinal fluid protein consisting of albumin and globulin in a ratio of about 5 to 1 varies between 15 mg. and 45 mg. per 100 ml. with an average of around 28 mg. per 100 ml.

Ventricular fluid contains between 5 mg. and 15 mg. per 100 ml., cisterna magna fluid between 15 mg. and 25 mg. per 100 ml., and lumbar fluid between 15 mg. and 45 mg. per 100 ml. The higher values in the lumbar fluid are believed to be a result largely of the relative stagnation occurring in that locality.

### Record of Physical Characteristics

State whether colorless, yellowish, reddish, greenish, grayish, and so forth, and whether perfectly clear, opalescent, faintly turbid, or purulent; describe the nature of any clot or sediment present.

### Cell Count

Use the same counting chamber and white blood cell counting pipet as for hematology, and make an accurate count of all red and white cells present. Unna's polychrome methylene blue stain is very satisfactory for use in distinguishing between white and red cells and in differentiating the types of white cells when their number is not great. It is prepared by dissolving 1 gm. of methylene blue and 1 gm. of potassium carbonate in 100 ml. of distilled water and ripening for immediate use if desired by heating at 60°C. for 10 minutes. The addition of a crystal of thymol will prevent the growth of molds. This stock solution should be kept in the refrigerator and filtered before using, when necessary. To insure an even distribution of any cells present, the fluid should be shaken thoroughly. Decant a small sample of the

fluid into a separate, clean, dry tube to avoid contaminating the main specimen with stain. The dye is drawn up to the 0.5 mark in the pipet used for counting white blood cells and the fluid is then drawn up to the 11 mark. This dilution produces a slight error which is negligible for clinical purposes. The fluid and the stain are shaken in the pipet for 1 minute. The counting chamber of 0.1 mm. depth, ordinarily used for counting of blood, is satisfactory for use with cerebrospinal fluid. When the fluid is stained as outlined, the nuclei of the white cells appear light blue and the cytoplasm is colorless and only faintly visible. The red blood cells appear slightly yellow. The whole ruled space of 9 square millimeters is counted, unless the cell count is so high that counting a smaller area will give an average value, which may be multiplied to give the count in the entire field. The total number of cells is multiplied by 10/9 to obtain the number per cubic millimeter, as the field is 9 square millimeters in area. For still greater accuracy and to overcome the slight error mentioned, this result can then be multiplied by 20/19 to correct for the dilution with stain.

Cerebrospinal fluid containing visible amounts of blood is not suitable for total cell counts because the leucocytes present result in counts that are too high. Even traces of blood may result in diagnostic errors in diseases in which the increase of leucocytes is between 20 and 100 cells per cubic millimeter.

When a more accurate differential determination of the white cells present is desired than is afforded by Unna's stain, a portion of the fluid is centrifuged at a moderate speed for several minutes, the supernatant fluid is decanted, and a drop of the sediment is smeared on a slide, allowed to dry, and stained with Wright's stain.

Standard Kahn (Diagnostic) Test with Spinal Fluid

In this test the spinal fluid globulins are precipitated by means of ammonium sulfate and redissolved in an amount of physiologic saline solution equivalent to one-tenth of the original spinal fluid volume. The concentrated globulin solution thus obtained is then examined with the standard antigen suspension.

Preparation of the Concentrated Globulin Solution

The reagents needed for the preparation of concentrated globulin solution are: (1) Spinal fluid, (2) 0.9% sodium chloride solution, and (3) saturated solution of ammonium sulfate of the highest purity ("reagent" quality). C.P. ammonium sulfate is not of sufficient purity for this test. Triple distilled water is employed in making this solution.

The following procedure is used:

1. Spinal fluid is centrifuged to render it free from cells and foreign particles. (If blood is present or fluid is cloudy, it should be so noted on the report. Further, if the Kahn test or Ross-Jones test for increased globulin is positive with

blood present in the fluid, the positive result might be due to the blood, so a new specimen free of blood should be requested and the tests repeated.)

2. 1.5 ml. of the clear fluid* is added to a standard Kahn test tube (75 mm. by 10 mm.).

3. To the same tube is added 1.5 ml. of a saturated solution of ammonium sulfate in the manner outlined in the following procedure for the Ross-Jones test for increased globulin (convenient to do at this point):

Ross-Jones test for increased globulin: This test is performed with a saturated solution of ammonium sulfate. A supersaturated solution is made by dissolving about 80 gm. of ammonium sulfate in 100 cc. of hot water. Some crystals will separate out on cooling; the solution is then known to be saturated. In performing this test in connection with the preparation of concentrated globulin solution for the spinal fluid Kahn, 1.5 ml. of the saturated ammonium sulfate is allowed to run in from a pipet held close to the bottom of the Kahn tube containing the 1.5 ml. of spinal fluid. The solution is underlaid carefully so that there will be no mixing. If globulin is present, a turbid white ring appears at the junction of the fluids within 2 minutes. If it appears immediately, the test is strongly positive. If it is not seen within 2 minutes, the test is negative. (Globulin is precipitated by a half-saturated ammonium sulfate solution. The point of half saturation is at the junction of the saturated solution of ammonium sulfate and the cerebrospinal fluid.) The test should not be read after 2 minutes. This test differs from the alcohol and Pandy tests in that it is specific for globulin and gives a positive reaction only in pathologic fluids.

4. After the test for increased globulin has been read, the fluids are mixed by covering the mouth of the tube with the thumb (protected with rubber) and shaking the tube back and forth vigorously. The mixture is placed in a 56°C. water bath for 15 minutes to hasten the precipitation of the globulins.

5. The mixture is centrifuged at high speed (about 2,000 rpm) for 15 minutes to throw down the precipitated globulins completely. The supernatant fluid should be water-clear. It will be noted that the amount of visible globulin precipitate in the bottom of the tube parallels the reading of the Ross-Jones test for increased globulin.

6. The supernatant fluid is removed as completely as possible with a finely drawn capillary pipet. An optional method for removing this fluid is to pour it off rapidly and invert the tube in a standard rack over clean filter paper, allowing the moisture that adheres to the tube to drain and be absorbed by the paper for a period of 10 minutes. This draining period is not necessary if the inside of the tube is wiped dry by means of filter paper. The filter paper is first wound around a glass rod or pencil and is then inserted into the tube without touching the precipitate. Fresh filter paper is employed for wiping each tube.

---

* If sufficient spinal fluid is available, 2.0 ml. of spinal fluid and 2.0 ml. of ammonium sulfate solution may be employed. If these quantities are used in the test, then 0.2 ml. of saline (instead of 0.15 ml.) should be added to the precipitated globulins.

The preferred method of removing the ammonium sulfate from the Kahn test tube in the performance of the Kahn test on spinal fluid is with the aid of a crooked, hollow piece of glass tubing. Put the Kahn test tube on the short end of the crooked tube, after the supernatant fluid has been poured off, and then immerse the Kahn test tube in a beaker of saline. Care must be taken so that the saline will not touch the globulin. After washing, dry the inside of the tube thoroughly and very carefully with a cotton swab, then turn the tube right side up and finish the test. (See Fig. 4.)

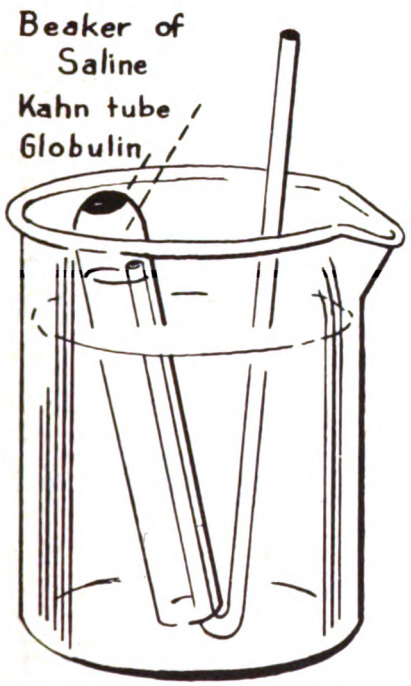



Fig. 4. Apparatus for removal of ammonium sulfate.

7. Saline solution (0.9 per cent) in the amount of 0.15 ml. is added to the globulin precipitate which is redissolved readily by gentle shaking. In adding this saline solution, the point of the pipet is held close to the bottom of the tube to avoid washing down traces of ammonium sulfate that may adhere to the inner wall. If the tube has been washed thoroughly by the crooked, hollow tube method, there will be no ammonium sulfate in the tube. In isolated instances, when an excess of globulin is present in the spinal fluid, the globulin precipitate may require somewhat more than 0.15 ml. of saline for complete solution.

8. This globulin solution should now be examined for clarity and for freedom from particles; it is then ready to be tested.

Preparation of Antigen Suspension

The saline solution is mixed with antigen in the same manner as for the standard test with serum - according to the antigen titer required for spinal fluid. The antigen suspension is allowed to stand 10 minutes and must be used in the test within the next 20 minutes.

Measuring Antigen Suspension

With a 0.2 ml. pipet graduated to 0.001 ml., 0.01 ml. of antigen suspension is measured to the bottom of a standard Kahn test tube.

Measuring Concentrated Globulin Solution

With another 0.2 ml. pipet, 0.15 ml. of the concentrated globulin solution is

measured into the antigen suspension tube. The rack is shaken vigorously for 10 seconds to mix the ingredients.

Controls Positive and negative spinal fluid controls are included.

Shaking After mixing the globulin solution with the antigen suspension, the test is shaken in a Kahn shaker at the standard speed for 4 minutes.

Addition of Saline Solution 0.5 ml. of physiologic saline solution is added to the test tubes.

Note: Add saline to one rack and complete reading before adding saline to the next rack.

Reading Results Four plus, three plus, and two plus reactions are reported as reactive, one plus reactions as weakly reactive, and plus-minus and negative reactions as non-reactive.

Quantitative Kahn Test with Spinal Fluid

Quantitative spinal fluid tests are performed on spinal fluids producing Reactive results in the Kahn standard test.

1. Prepare dilutions of spinal fluid as indicated in Table 1.

2. Prepare standard antigen suspension as described under "Kahn Standard Qualitative Test With Serum."

3. Place thumb over mouth of mixing vial and shake gently to suspend.

Table 1. Spinal Fluid Dilution

| Tube | Spinal Fluid[1] | Saline solution | Designated dilution |
|---|---|---|---|
| | ml. | ml. | |
| 1 ..... | Quantity available | None | 1:10 |
| 2 ..... | 0.2 | 0.1 | 1:15 |
| 3 ..... | 0.2 | 0.2 | 1:20 |
| 4 ..... | 0.1 | 0.2 | 1:30 |
| 5 ..... | 0.1 | 0.3 | 1:40 |

[1] Whole spinal fluid is considered a 1:10 dilution since the qualitative test is performed on spinal fluid globulin concentrated 10 times.

4. Pipet 0.01 ml. of the antigen suspension directly to the bottom of a Kahn test tube. One tube is required for each dilution of spinal fluid being tested.

5. Add 0.15 ml. of diluted spinal fluid to each tube, starting with the highest dilution.

6. Shake rack by hand for 10 seconds.

7. Shake rack on Kahn shaker for 4 minutes.

8. Remove rack from shaker, add 0.5 ml. of saline solution to each tube, mix, and read immediately.

9. Note the highest dilution of spinal fluid producing a Reactive result (4 ≠, 3 ≠, or 2 ≠).

10. Calculate the Kahn units according to the formula S = 4D, where S is the potency of the spinal fluid in terms of Kahn units, and D is the highest dilution in which a 4 ≠, 3 ≠, or 2 ≠ reaction is observed.

Example:

a. Spinal fluid Reactive at 1:10 dilution (designated) 10 x 4 = 40 Kahn units.
b. Spinal fluid Reactive at 1:40 dilution (designated) 40 x 4 = 160 Kahn units.

## TESTS FOR INCREASED GLOBULIN

### Ross-Jones Test

This is performed during the Kahn procedure.

### Pandy's Test

Pandy's Reagent is approximately a 10 per cent aqueous phenol solution. Transfer approximately 1 ml. of Pandy's reagent to a test tube and add 1 drop of spinal fluid. An increase of globulin is indicated by the appearance of a bluish-white cloud. The density of the cloud is reported as +, ++, +++, or ++++, roughly measuring the degree of increase in globulin. (Either test will give a positive reaction with normal spinal fluid contaminated with blood.)

### Colloidal Gold Reaction

Lange's colloidal gold test is performed by mixing cerebrospinal fluid in varying proportions with a colloidal suspension of gold chloride. Reactions are indicated by a color change. The test should not be conducted on cerebrospinal fluid containing blood because false positive and variable reactions may be produced. Normal spinal fluids produce no precipitation of the gold solution and the color remains orange pink. Complete precipitation produces a colorless solution and intervening degrees of precipitation range through a color change of red, lilac, and blue. Degrees of precipitation or color change are indicated by numbers as follows:

5 - Colorless, complete precipitation
4 - Pale blue
3 - Blue
2 - Purple or lilac
1 - Red or bluish red
0 - Orange pink, no precipitation

The test has no specific diagnostic importance. Many pathologic conditions of the central nervous system produce no change. Many cases of syphilis of the central nervous system and inflammatory conditions of the meninges produce marked changes, but they are of diagnostic importance only insofar as they indicate abnormality of the protein content of the fluid. The terms "paretic," "syphilitic," and "meningitic" curves which formerly were used to describe these changes have been discarded for the terms "first zone," "mid-zone," and "end zone" reactions.

## Reagents

### Colloidal Gold Solution

Standard colloidal gold solution is prepared for naval medical activities at the United States Naval Medical School and may be obtained upon written request.

Each colloidal gold solution has its own specific acid solution and this cannot be interchanged with that of another lot. For preparing this solution, sufficient alkaline colloidal gold for immediate use is removed from the stock container. To each 30 ml. (amount sufficient for one rack), add the indicated amount of the accompanying standard acid solution. Acid should only be added just prior to use. Do not add acid to the bottle of stock solution.

### Saline Solution

The solution used is an accurately prepared 0.4 per cent sodium chloride solution. This should be prepared by dissolving 4.0 gm. of anhydrous reagent grade sodium chloride (accurately weighed) in redistilled water and diluting to a volume of 1,000 ml. in a volumetric flask.

## Materials

### Test Tubes

The tubes used are 15 by 100 mm. in size. Special precautions to insure chemical cleanliness are necessary. The test tubes should be washed with soap and water, rinsed in tap and distilled water and dried, following which they are placed in aqua regia. (This is one part of nitric acid and three parts of hydrochloric acid. This potent mixture should be kept under a hood with the additional precaution to keep the lid slightly ajar when the mixture is first made as the potent fumes may blow off the glass cover. Make the mixture in a glass container). See Washing of Glassware for further steps in cleaning these tubes.

### Pipets

Ten ml. pipets graduated to 0.1 ml. and 1 ml. pipets graduated to 0.01 ml. are used.

## Procedure

1. Place 11 clean, dry test tubes in a row in a rack.

2. Add 1.8 ml. of saline solution (0.4 per cent sodium chloride) to the first tube and 1.0 ml. to each of the other 10 test tubes.

3. Add 0.2 ml. of cerebrospinal fluid to the first tube and mix thoroughly. This makes a 1 to 10 dilution. Transfer 1.0 ml. from the first tube to the second tube, making a 1 to 20 dilution. The process is continued down through the tenth tube from which 1.0 ml. is discarded. The dilutions are 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1,280, 1:2,560, and 1:5,120. The eleventh tube is used as a control.

4. Add 5 ml. of gold solution to each tube. Shake well, cover, and let stand for 24 hours at room temperature.

Modified Colloidal Gold Test

For economy, the procedure may be carried out with one-half the amounts mentioned.

1. Place 11 chemically clean test tubes in a rack.

2. Into the first tube place 1.8 ml. of 0.4 per cent sodium chloride solution and 0.5 ml. in each of the remaining 10 tubes.

3. Add 0.2 ml. of spinal fluid to the first tube and thoroughly mix. (The dilution will be more accurate if 0.2 ml. of fluid is diluted with 1.8 ml. of saline solution rather than 0.1 ml. of fluid with 0.9 ml. of saline solution. If the latter is used, the 1 ml. is not discarded from the first tube as directed in step 4.)

4. Discard 1 ml. from the first tube, then transfer 0.5 ml. to tube 2; mix thoroughly and remove 0.5 ml. and place in tube 3; continue until the tenth tube is reached and then discard 0.5 ml. from this tube. The eleventh tube is used as a control.

5. Add to each tube 2.5 ml. of colloidal gold solution.

6. Mix thoroughly and set aside for 24 hours. The readings are now made and recorded in the same way as in the original test.

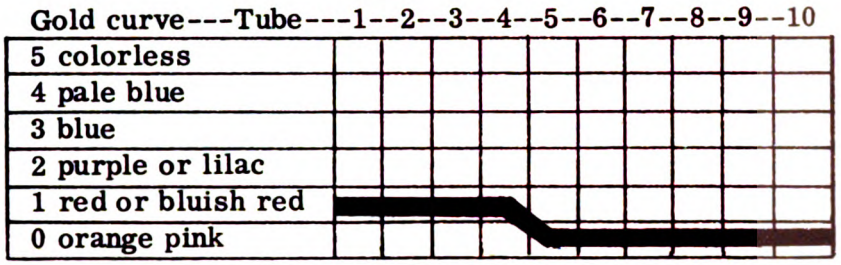
| Gold curve---Tube---1--2--3--4--5--6--7--8--9--10 | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 5 colorless | | | | | | | | | | |
| 4 pale blue | | | | | | | | | | |
| 3 blue | | | | | | | | | | |
| 2 purple or lilac | | | | | | | | | | |
| 1 red or bluish red | | | | | | | | | | |
| 0 orange pink | | | | | | | | | | |

Interpretations

Any color change less than "lilac" may be considered of no significance. Any

color change greater than "lilac" should be considered as definite evidence of an abnormal fluid. Color changes to and including "lilac" are seen in many normal fluids.

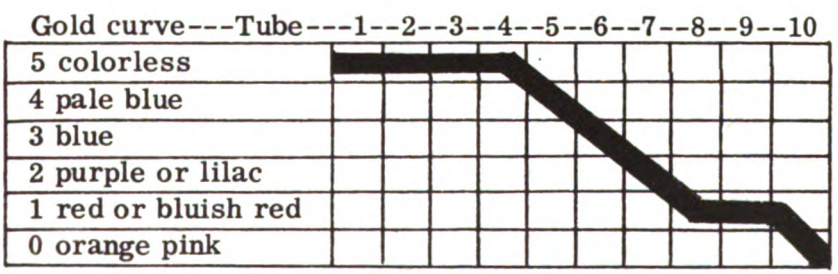
Typical Normal Curve




First Zone Curves

These are the reactions showing partial or complete precipitation in the first few test tubes. They occur so frequently in spinal fluids of patients with syphilis of the central nervous system and multiple sclerosis that they are almost of diagnostic importance. They are seen in about 80 per cent of patients with paresis, 40 per cent with syphilitic meningitis, 8 per cent with tabes dorsalis, 15 per cent with cerebrovascular neurosyphilis, 22 per cent with syphilis of the spinal cord, 9 per cent with asymptomatic neurosyphilis and in practically all cases of syphilitic optic atrophy.

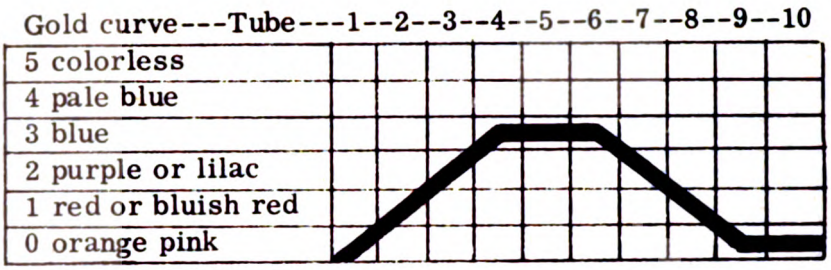
Typical First Zone Curve




Mid-Zone Curves

In these reactions the greatest change is noted in the fourth and sixth tubes. They rarely are of a degree to produce complete precipitation. They may be found in any abnormal fluid, but their presence is of no differential diagnostic significance.

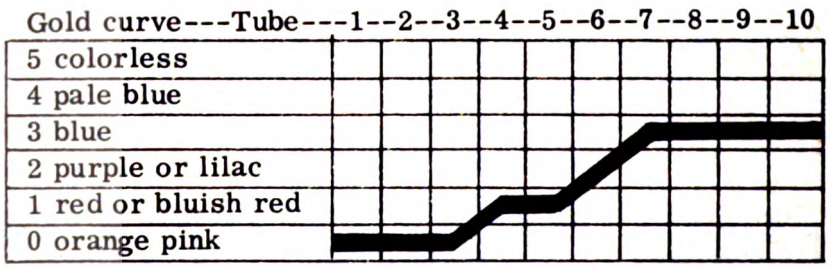
## Typical Mid-Zone Curve




### End Zone Curves

The greatest changes are to be seen in the sixth to the tenth test tubes, but they rarely show complete precipitation. This type of curve is seen in spinal fluids with a very high protein content and especially when the albumin-globulin ratio is high. Such conditions are acute purulent meningitis, subarachnoid block, and hemorrhage into the subarachnoid space.

## Typical End Zone Curve



## SECTION X

## PREPARATION OF SEROLOGIC SPECIMENS FOR SHIPMENT

### Use of Merthiolate as a Preservative

Spinal fluids and serums which have become grossly contaminated in transit are unsatisfactory for serologic tests for syphilis. Some of the products of bacterial metabolism are soluble and changes of the original components of the body fluids occur. Any serologic results obtained, therefore, are of questionable value.

Although spinal fluid and blood are usually drawn with reasonable attention to sterility, many specimens are contaminated when they are received in the laboratory.

The use of merthiolate (sodium ethylmercurithiosalicylate)* as a bacteriostatic agent for spinal fluid has been reported.** It does not interfere with the mechanisms of the serologic tests nor introduce a dilution factor.

### Preparation of Merthiolate Solution

On the day to be used, prepare the necessary amount of merthiolate solution by adding 1.0 gm. of merthiolate to each 100 ml. of distilled water. Do not use commercially prepared tinctures or solutions.

### Preparation of Collection Tubes for Spinal Fluid for Routine Serology

1. Pipet 0.1 ml. of 1 per cent aqueous merthiolate solution to the bottom of each 13 mm. by 100 mm. tube.

2. Place the tubes in a vacuum desiccator over calcium chloride at room temperature protected from the light. In 24 hours, dehydration is usually complete if a satisfactory vacuum has been established.

3. For stoppers, submerge corks in hot (not smoking) paraffin for 1 minute, and while hot, roll on a cloth to remove excess paraffin.

4. Remove the tubes from the desiccator and stopper tightly with paraffin corks.

5. Store the tubes in the dark. They will remain usable for months.

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* "Armed Services Catalog of Medical Materiel," No. 1-285-675 (10 gm. for preserving blood serum and vaccines).

** Harris, A., and Mahoney, J.F.: "Merthiolate as an Effective Bacteriostatic Agent in Spinal Fluid Specimens," J. Ven. Dis. Inform., pp. 25, 46, Feb. 1944.

The concentration of merthiolate obtained when 2.0 ml. to 8.0 ml. of spinal fluid is added to each tube is sufficient to inhibit bacterial growth in transit.

Preparation of Collection Tubes for Serum for Routine Serology

Follow the same technique as for spinal fluid except use 12 mm. by 75 mm. tubes. One mg. of merthiolate is suitable for shipment of 2 ml. to 4 ml. of serum.

Preparation of Serum for TPI Test

All the equipment used to obtain the blood specimen must be scrupulously clean (free of acid and alkali) and sterile. See Section II "Obtaining a Clear Specimen" under Venipuncture. A sterile paraffined cork should be used as a closure for the tube, or a sterile screw cap, because rubber contains a substance which is TOXIC to treponemes. Shipment in a vaculator tube is harmful for the same reason.

## SECTION XI

## DAVIDSOHN TEST FOR INFECTIOUS MONONUCLEOSIS

The Davidsohn test for infectious mononucleosis is used routinely at the United States Naval Medical School. It consists of two parts, the presumptive and the differential test. The presumptive test is performed first. In certain circumstances, as noted under Indications, the differential test "absorption with guinea pig kidney antigen" is essential. With elevated titers of antisheep agglutinins, when the diagnosis is still in doubt, a second differential test "absorption with beef cell antigen" should be performed.

### Presumptive Davidsohn Test

#### Principle

The test is based on the agglutination of sheep erythrocytes by the heterophilic antibodies in the serum of patients with infectious mononucleosis.

#### Indication

The presumptive test is indicated in cases where the clinical or the hematologic findings or both suggest infectious mononucleosis.

#### Materials

1. Test tubes, 75 mm. by 10 mm.

2. Serum inactivated for 30 minutes at 56°C.

3. Two per cent suspension in saline of sheep red cells washed three times in physiologic solution of sodium chloride. The sheep red cells should be not less than 24 hours old and not older than 1 week. They should be used only if the third washing gives a perfectly colorless supernate. The cells should be washed and the suspension prepared and used on the same day.

#### Procedure

1. Set up a row of 10 tubes.

2. Add 0.4 ml. of physiologic solution of sodium chloride to the first tube and 0.25 ml. to the other tubes.

3. Add 0.1 ml. of serum to the first tube, mix and transfer 0.25 ml. to the second tube, and so on, until the last tube is reached. Mix and discard 0.25 ml. from the last tube. Serum dilutions are 1:5, 1:10, 1:20, and so forth.

4. Add 0.1 ml. of 2 per cent suspension of sheep red cells. Shake the tubes. Final dilutions are 1:7, 1:14, and so on.

5. Let stand at room temperature. When speed is indicated, the reading may be done after 15 minutes. If the result is positive (agglutination in dilutions 1:224 or higher), the test may be considered completed except that the final titer will be higher after 2 hours' incubation. If negative (titer less than 1:224), repeat the reading at intervals as frequent as convenient. Final negative result (titer less than 1:56) should not be recorded until after 2 hours' incubation. If speed is not a factor, it may be more convenient to read results at the end of 2 hours.

6. Results are read after shaking the test tubes to resuspend the sediment. Check with the naked eye. If no clumping is visible, place the tube horizontally on the stage of the microscope and read with a low power objective (scanning lens - e.g., 25 mm. or 35 mm.) permitting viewing of a test tube.

Interpretation

In the presence of clinical and/or hematologic findings suggestive of infectious mononucleosis, titers of 1:224 or higher in the presumptive test confirm the diagnosis of infectious mononucleosis. If the titer of the presumptive test is less than 1:224 in the presence of clinical and/or hematologic findings suggestive of infectious mononucleosis; or, if the titer of the presumptive test is 1:224 or higher in the absence of clinical and hematologic findings suggestive of infectious mononucleosis; or, if the patient has a history of a recent horse serum injection, the result of the presumptive test should be checked by the differential test (see below).

Differential Davidsohn Test

Principle

The heterophilic antibodies (antisheep agglutinins) in infectious mononucleosis are not of the Forssman type. They are not absorbed by suspensions of guinea pig kidney but are readily and completely absorbed by beef red cells. The heterophilic antibodies in normal persons, in horse serum sensitization, and in a variety of other conditions are of the Forssman type and are readily and completely absorbed by suspensions of guinea pig kidney. In horse serum sensitization absorption with beef red cells removes the sheep agglutinins readily and completely, whereas in normal persons and in patients with diseases other than infectious mononucleosis or horse serum sensitization, sheep agglutinins are frequently removed only partially by the beef cell antigen.

## Indications for Differential Test Absorption with Guinea Pig Kidney Antigen

These include:

1. A titer of antisheep agglutinins (1:112 or less), as determined by the presumptive test, in cases suspected of having infectious mononucleosis.

2. A titer of antisheep agglutinins of 1:56 or higher in patients without clinical or hematologic findings of infectious mononucleosis.

3. A history of a recent injection of a horse serum in a patient with a titer of antisheep agglutinins of 1:56 or higher as determined by the presumptive test.

## Technique of Differential Test with Guinea Pig Kidney Antigen

### Materials

1. Test tubes for absorption, 85 mm. by 12 mm., and tubes for agglutination tests, 75 mm. by 10 mm.

2. Serum inactivated for 30 minutes at 56°C.

3. Two per cent suspension in saline of washed sheep red cells.

4. Guinea pig antigen.

### Preparation of Guinea Pig Antigen

Guinea pig kidneys are stored frozen until needed, then thawed and washed with physiologic saline until the washings are free of blood. They are then mashed into a fine pulp and made into 20 per cent suspension in physiologic saline. The suspension is boiled 1 hour in the water bath and loss by evaporation is made up with distilled water. Phenol is added to make a 0.5 per cent solution. The antigen is stable for many months when kept in the refrigerator.

### Procedure for Absorption with Guinea Pig Antigen

1. Place in a test tube (85 mm. by 13 mm.) 1 ml. of the thoroughly shaken suspension of guinea pig antigen.

2. Add 0.2 ml. of inactivated serum.

3. Shake well and let stand for 3 minutes.

4. Centrifuge at 1,500 rpm for 10 minutes.

5. Remove the supernatant fluid carefully with a capillary pipet. Make sure to transfer only the clear supernate without particles.

6. Set up a row of 10 tubes (75 mm. by 10 mm.). Add 0.25 ml. of physiologic solution of sodium chloride to all tubes except the first.

7. Add 0.25 ml. of the supernatant fluid to the first tube.

8. Add 0.25 ml. of the supernatant fluid to the second. Mix and transfer 0.25 ml. to the third tube, and so on. Discard 0.25 ml. from the last tube. The serum dilutions are 1:5, 1:10, 1:20, and so forth.

9. Add 0.1 ml. of a 2 per cent suspension of sheep cells. Final dilutions are 1:7, 1:14, 1:28, and so on. Shake.

10. Let stand at room temperature. When speed is indicated, the reading may be done after 15 minutes. If the test is positive, which means agglutination in the same dilution as the presumptive test or in not more than three dilutions or tubes below that of the titer of the presumptive test, then the test can be reported as positive for infectious mononucleosis. If the result is negative, which means more than three tubes difference in the agglutination as compared with the titer of the presumptive test, repeat the reading at intervals as frequent as convenient. Final negative results should not be recorded until after 2 hours' incubation.

11. Results are read after shaking the test tubes to resuspend the sediment. Check with the naked eye. If no clumping is visible, place the tube horizontally on the stage of the microscope and read with a low power objective (scanning lens - e.g., 25 mm. or 35 mm.) permitting viewing of a test tube.

Interpretation of Differential Test with Guinea Pig Kidney Antigen

The differential test for infectious mononucleosis is positive if the titer of antisheep agglutinins after absorption with guinea pig kidney is not more than three tubes lower than the titer of the presumptive test.

Examples:

Table 1. Reporting Results

| Titer | | Results |
|---|---|---|
| Presumptive Test | Differential test after absorption with guinea pig kidney | |
| 1:224 | 1:112 | Positive for infectious mononucleosis |
| " | 1:56 | " " " " |
| " | 1:28 | " " " " |
| " | 1:14 | Negative for infectious mononucleosis |
| " | 1:7 | " " " " |
| 1:56 | 1:56 | Positive for infectious mononucleosis |
| " | 1:28 | " " " " |
| " | 1:14 | " " " " |
| " | 1:7 | " " " " |
| 1:28 | 1:28 | Positive for infectious mononucleosis |
| " | 1:14 | " " " " |

In all positive tests the absorption with beef cells, if done, will show complete or almost complete removal of agglutinins. In the negative tests absorption with beef cells, if done, may show incomplete removal of the agglutinins.

## Indications for Differential Test Absorption with Beef Cell Antigen

It may be preferable to do the absorption with guinea pig kidney and with beef cell antigens in every case. The absorption with beef cells is essential in cases of patients with elevated titers of antisheep agglutinins if no clinical or hematologic findings are present suggestive of infectious mononucleosis. In most of these instances the antisheep agglutinins will be completely removed by guinea pig kidney antigen, but, in rare instances, the absorption may be incomplete. The use of the beef cell antigen will then be decisive.

## Technique of Differential Test with Beef Cell Antigen

### Materials

1. Test tubes for absorption, 85 mm. by 13 mm., and tubes for agglutination tests, 75 mm. by 10 mm.

2. Serum inactivated for 30 minutes at 56° C.

3. Two per cent suspension in saline of washed sheep red cells.

4. Beef cell antigen.

Preparation of Beef Cell Antigen

1. Beef cells are washed three times with physiologic saline and packed by centrifuging. One volume of packed cells is suspended in four volumes of saline and the suspension is boiled in the water bath for 1 hour. Loss by evaporation is made up with distilled water. Phenol is added to make a 0.5 per cent solution. The antigen is stable for many months when kept in the refrigerator.

Procedure for Absorption with Beef Cell Antigen

1. Place in a test tube (85 mm. by 13 mm.) 1 ml. of the thoroughly shaken suspension of beef cell antigen.

2. Add 0.2 ml. of inactivated serum.

3. Shake well and let stand for 3 minutes.

4. Centrifuge at 1,500 rpm for 10 minutes.

5. Remove the supernatant fluid carefully with a capillary pipet. Make sure to transfer only clear supernate without particles.

6. Set up a row of 10 tubes (75 mm. by 10 mm.). Add 0.25 ml. of physiologic solution of sodium chloride to all tubes except the first.

7. Add 0.25 ml. of the supernatant fluid to the first tube.

8. Add 0.25 ml. of the supernatant fluid to the second. Mix and transfer 0.25 ml. to the third tube, and so on. Discard 0.25 ml. from the last tube. The serum dilutions are 1:5, 1:10, 1:20, and so forth.

9. Add 0.1 ml. of a 2 per cent suspension of sheep cells. Final dilutions are 1:7, 1:14, 1:28, and so forth. Shake.

10. Let stand at room temperature. When speed is indicated, the reading may be done after 15 minutes. If agglutination is present in the same dilution as in the presumptive test or in dilutions from two to three tubes lower, the result is negative for infectious mononucleosis. If no agglutination is present, repeat the reading at intervals as frequent as convenient. If no agglutination is present, final results should not be recorded until after 2 hours' incubation.

11. Results are read after shaking the test tubes to resuspend the sediment. Check with the naked eye. If no clumping is visible, place the tube horizontally on the stage of the microscope and read with a low power objective (scanning lens - e.g., 25 mm. or 35 mm.) permitting viewing of a test tube.

## Interpretation of Differential Test with Beef Cell Antigen

The test for infectious mononucleosis is positive in a serum in which the absorption with guinea pig kidney failed to remove the antisheep agglutinins completely and in which the beef cell antigen completely removed the antisheep agglutinins.

Examples:

Table 2. Reporting Results

| Titer | | | Result |
|---|---|---|---|
| Presumptive Test | Differential test after absorption with: | | |
| | guinea pig kidney | beef red cells | |
| (a) 1:224 | 0 | 1:112 | Negative for infectious mononucleosis |
| (b) 1:448 | 1:224 | 1:224 | Negative for infectious mononucleosis |
| (c) 1:448 | 1:224 | 0 | Positive for infectious mononucleosis |

## SUMMARY

The presumptive test for infectious mononucleosis is a quantitative nonspecific test. Titers of 1:224 or higher make possible a presumptive diagnosis of infectious mononucleosis in the presence of clinical and/or hematologic findings suggestive of the disease.

The differential test consists of determination of the antisheep agglutins after (1) absorption with guinea pig antigen and (2) absorption with beef cell antigen.

The differential test is specific for infectious mononucleosis. A positive test is indicated by (1) incomplete removal of antisheep agglutinins by guinea pig kidney and (2) complete removal of the antisheep agglutinins by beef cells.

## SECTION XII

## TEST FOR COLD AGGLUTININS

The serum of certain individuals may agglutinate homologous-type cells when the two are mixed and kept at refrigerator temperatures or sometimes even at room temperature. This phenomenon is due to so-called cold agglutinins. These cold agglutinins are nonspecific and clump cells regardless of the blood group. When cells of the same person are clumped by his own serum, they are called auto-agglutinins. The cell clumps break up when the mixtures are brought to body temperature. Cold agglutins are found in a considerable number of cases of primary atypical pneumonia and less frequently in other acute infections and certain pathologic conditions.

### Materials

1. Standard Kahn test tubes.

2. Saline solution, 0.85 per cent.

3. Sodium citrate solution, 2.5 per cent.

4. Patient's cells and serum.

### Collection of Blood Specimen

As most cold agglutinins are also auto-agglutinins, extreme care must be used in preparing the serum for the test.

1. A cold syringe should not be used to draw the blood specimen. The syringe may be warmed by enclosing it in the palm of the hand for a few minutes.

2. The tube to receive the blood should be warm.

3. After the blood specimen is obtained, it must be kept warm while in the process of delivering it to the laboratory. This can be accomplished by transporting the tube of blood in a glass of warm water, or the technician may carry the tube of blood in his shirt pocket.

4. The laboratory technician should place the tube immediately in a 37°C. water bath or incubator until it is centrifuged.

5. After the serum is separated from the cells, it should be stored in a refrigerator until the test is set up. If the specimen is chilled before the serum is separated the cells may absorb the cold agglutinins from the serum, and in centrifuging, the

cold agglutinins will be partly or completely removed with the packed cells or clot.

Procedure.

1. Collect approximately 7 ml. of blood by venipuncture.

2. Six drops of the blood (from the needle with the bevel down) are added to a tube containing 10 ml. of a 2.5 per cent sodium citrate solution. Mix by inverting the tube.

3. Place about 5 ml. of blood in a clean, dry test tube and allow to clot. When the clot has formed, centrifuge and separate the serum. Note: The serum must be fresh because old serum may give falsely negative results.

4. Centrifuge the patient's cells for 15 minutes at 2,000 rpm in a graduated centrifuge tube, pour off the supernatant fluid, and then make a 2 per cent suspension in 0.85 per cent saline solution.

(Type O cells from a healthy individual may be prepared as above and used in place of the patient's cells.)

5. Place 11 small test tubes (Kahn) in a rack.

6. Add 1.5 ml. of 0.85 per cent saline to the first tube and 1 ml. to each of the remaining 10 tubes.

7. Add 0.5 ml. of the patient's serum to the first tube. Mix thoroughly and transfer 1 ml. to the second tube, mix and transfer 1 ml. to the third tube, and so on to the tenth tube from which 1 ml. is discarded. The eleventh tube is a control and contains no serum.

8. Add to each tube 0.1 ml. of the previously prepared cell suspension (step 4). After shaking each tube to insure thorough mixing the rack is placed in an ice bath, which is in turn placed in a refrigerator overnight.

Reading of Results

Agglutination, if present, will disappear in a few minutes at room temperature, so the reading must be made immediately.

1. Remove one tube at a time from the ice bath and examine for agglutination. Report the titer as the highest dilution showing agglutination of one plus or more. The serum dilution in the first tube is 1:4, in the second tube 1:8, and so on.

2. If there is no agglutination in any of the tubes, the test is reported as negative.

3. If positive, allow the tubes to stand at room temperature for an hour and then make a second reading. If the reaction is a true cold agglutination, the clumps will disperse.

Interpretation

A titer of 1:32 or higher is suggestive of atypical or virus pneumonia. A titer of 1:1,024 or above is not uncommon in that disease. Elevated titers of agglutinins may also occur in other conditions, such as acquired hemolytic jaundice, cirrhosis and other diseases of the liver, chronic sepsis, leishmaniasis, and blackwater fever.


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